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Immunological Approaches to the Diagnosis of Systemic Candidiasis

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IMMUNOLOGICAL APPROACHES TO THE DIAGNOSIS OF SYSTEMIC CANDIDIASIS

by

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The increasing incidence of systemic candidiasis and the difficulties with diagnosing the disease on the basis of clinical data and culture results have prompted investigation into immunological methods of diagnosis. Detection of anti-Candida antibodies has proved to be not sufficiently specific for diagnosis of systemic disease since antibodies are frequently found in the normal individual in whom the organism lives as a commensal and in superficially infected individuals. Our project resulted in the development of a new quantitative immunofluorescence assay for quantitation of anti-Candida antibodies which is able to differentiate quite well ($p < 0.0005$) between systemically infected patients and normals or superficially infected patients. We have also investigated the value of purified antigens and have shown that the use of cytoplasmic protein antigens rather than those containing cell wall polysaccharide confers greater specificity on a qualitative test for anti-Candida antibodies but that a carbohydrate-containing antigen is adequate for differentiating systemic disease when a quantitative test is used.

Attempts to diagnose systemic candidiasis by detection

of circulating antigen have met with varying degrees of success. We were unable to detect antigen by counterimmunoelectrophoresis, a quantitative immunofluorescence inhibition assay, or an ELISA inhibition assay in the sera of rabbits in whom a Candida endocarditis was induced or in the sera of systemically infected patients. One postulated reason for this failure is the presence of the antigen in the form of circulating immune complexes. We have, therefore, investigated the possibility of the existence of complexes containing Candida antigen and antibody in the sera of patients with systemic candidiasis. Using four screening tests, a statistically significant difference ($p < 0.05$) was found for immune complex levels in a group of patients and a group of normal individuals. Isolation and characterization of immune complex-containing fractions from five patients with candidiasis indicated the presence of anti-Candida antibodies and/or antigen in at least three of them. Therefore, these fungal immune complexes can be assumed to exist and may play a role in the difficulties in detecting circulating antigen and, furthermore, may be involved in the pathogenesis and progression of disseminated candidiasis.

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Introduction

The clinical conditions now known to be caused by the organism Candida albicans have been recognized for centuries. Hippocrates described "aphthae" or white patches in debilitated patients in his "Epidemics" and the disease "thrush" was noted in Pepys' diary in 1665 (1). The oral lesion was recognized early as a condition of the newborn as well as of the debilitated and Veron in 1835 postulated that it was acquired during passage through the womb. Vaginal candidiasis was first described by Wilkinson in 1849 and systemic disease, acquired by hematogenous spread, was described by Zenker in 1861 (2).

In 1839, Langenbeck (3) described a fungus in aphthae but he did not consider the fungus to be related to the disease. However, Berg in 1841 and Bennett (4) in 1844 conclusively demonstrated the fungal etiology of thrush. Bennett recognized the dimorphic nature of the organism and in 1847 Robin placed it in the genus Oidium under the name Oidium albicans (2). He described in detail the egg-shaped, oval yeast cell and the mycelial form of the fungus. In 1890, Zopf described a similar organism as Monilia albicans bringing confusion to the nomenclature of the yeast (2). Finally, in 1923 Berkhout cleared up the confu-

sion in assigning the generic designation Candida to those fungi which developed a pseudomycelium and reproduced by budding (5).

Today, 81 species of Candida have been recognized. Most live as commensals in humans, representing part of the normal flora of cutaneous and mucocutaneous areas. The most commonly encountered pathogenic species is Candida albicans. It is a small oval, budding, yeast-like fungus, 2.5 by 4 by 6 μm, which develops a pseudomycelium by elongation of cells which fail to detach (6). In sputum, tissue and exudates both the budding cells and fragments of pseudomycelia may be seen. Other species including C. parapsilos, C. tropicalis, C. stellatoidea, and C. guillermondi, are of more limited pathogenicity, although they may be isolated in some of the less common but frequently more severe clinical manifestations of candidiasis.

Candida albicans is a normal inhabitant of the alimentary tract and the mucocutaneous regions in man. It is regularly present in small numbers in the mouths of normal healthy adults and can be found in the normal vagina of healthy non-pregnant women with an incidence of about 5% (7). The gastro-intestinal tract has a small but constant population of C. albicans, the number being influenced by the diet of the individual and by the presence of other members of the intestinal flora. Normal skin less commonly harbors a resident flora of C. albicans, but almost any

damage to skin or environmental change leads to rapid colonization (8).

In the normal host, control is maintained such that the number of organisms inhabiting these sites remains small and clinically insignificant. The balance between commensal and host, however, is delicate and can be easily upset leading to overgrowth and to potential pathogenicity of the organism. Poor oral hygiene or even a small amount of antibiotics may promote an increase in the number of organisms in the mouth. In the adult this is usually without untoward results. In the newborn, however, before an oral ecology is established and where a low pH in the mouth may promote proliferation of the organism, even a few organisms presage clinical thrush (9). In older children, chronic thrush usually indicates polyendocrine disturbances or an underlying defect in natural defenses. In adults, significant oral infection may occur as a result of mild avitaminosis or as a complication of diabetes mellitus, advanced neoplasia, or the administration of steroids, antibiotics, or other drugs (9).

In gravid women there is a distinct increase in clinical Candida vaginitis. Pregnancy appears to affect the carbohydrate content of the vagina and this promotes overgrowth of the organism. Diabetes and antibiotic therapy may also predispose to vaginal candidiasis. Candida balanitis or balanoposthitis may occur rarely in males, most often where there is a history of vaginitis in the

spouse and the condition is probably a conjugal infection (9).

It has been established that other members of the intestinal flora exert control over the population of C. albicans in the lower intestinal tract through a number of mechanisms such as production of inhibitory concentrations of lactic acid and competition for available nutrients (1). Overgrowth of yeasts may occur in the young before a balanced flora is established and may be associated with clinical symptoms such as a diaper rash following perianal colonization (9). In adults in whom orally administered antibiotics have altered the intestinal flora, overgrowth of the organism may lead to an irritating pruritis ani, destruction of the esophageal mucous membrane, or invasion of the gastro-intestinal mucosa. Other factors promoting gastro-intestinal overgrowth of the organism are diabetes, corticosteroid administration, irradiation, various neoplasias, blood dyscrasias, endocrinopathies and other debilitating conditions (10).

Overgrowth of C. albicans on the skin frequently involves intertriginous areas such as the axillae, groin, intermammary fold, intergluteal folds, and interdigital spaces. It may occur as a result of metabolic disorders or environmental conditions such as moisture, occlusion, or maceration of the skin. Chronic mucocutaneous candidiasis occurs in patients with genetic defects in the immune response and in patients with polyendocrine disorders (9).

Besides these localized overgrowths of Candida in sites where the organism normally lives as a commensal, a more significant imbalance in the forces which normally prevent transition from commensalism to parasitism can lead to dissemination of the organism to multiple internal sites and to a potentially lethal systemic infection. Developments in medicine since 1940 have greatly increased the occurrence of this imbalance of forces and the subsequent severe fungal infections.

With the onset of widespread use of antibiotics, in particular broad-spectrum antibiotics, the delicate ecosystem of which Candida is a member may frequently be disturbed (10). Not only do antibiotics suppress the normal flora which acts as competition for the commensal Candida organisms, but, in addition, some damaging action by antibiotics on the outer layers of mucous membranes, notably of the intestinal tract, may facilitate the penetration of the organism. Should the host's resistance be impaired, further invasion, dissemination, and severe clinical symptoms may occur (11).

The use of steroid therapy, immunosuppressive drugs, antimetabolites, and cytostatic drugs also affects the balance between host and saprophytic organism. All of these treatment modalities, which can improve the patient's prognosis with respect to other disorders, may cause a significant abrogation of the body's defenses against invading or proliferating fungal organisms. Normally, the phagocy-

tic cells of the body are capable of ingesting Candida and the cellular response to the invader is considered the most important component of the defense. The organs of the body which have large numbers of histiocytes, for instance, lungs, liver, and spleen, are relatively resistent to infection with Candida. On the other hand, there is evidence that when deep-seated Candida infections do occur, the cellular response is often sluggish or lacking. Therefore, therapy with those agents which decrease the numbers or effectiveness of phagocytic cells in the body can lead to systemic infection and dangerous clinical symptomatology (12).

The practice of modern medicine has seen a tremendous increase in the number and extent of invasive techniques undertaken to provide the patient with greater length and quality of life. The common use of intravenous catheters provides an easy entry into the body for organisms which are normally found on the skin (13). Hemodialysis and peritoneal dialysis are procedures which also breach the body's non-specific defenses and allow entry of normally non-pathogenic agents. One important source of entry seems to be the heart-lung machine, where Candida cells may easily hide and escape the procedures of disinfection so that on use of the machine, the fungi enter the systemic circulation of the patient (14). Postoperative antibiotic prophylaxis after heart surgery can increase further the likelihood that the invading Candida will cause dissemina-

commensal Candida and the host has been upset and the fungus becomes invasive, the results can be disastrous for the host. The disease, when untreated, can be rapidly fatal leading to death within a few days (18). Taschdjian et al., in a review, found the overall mortality rate in eight studies containing 141 patients to be 75% (3).

Unfortunately, the advances in medicine which have brought about the increased incidence of severe fungal infections have not been accompanied by advances in the treatment of these infections. Drugs with a relatively high therapeutic index, such as Ketoconazole and Mycostatin, have been developed for the treatment of mucocutaneous infections; however, the drug of choice for disseminated candidiasis is Amphotericin B which has a large number and variety of untoward effects. These include anaphylaxis, thrombocytopenia, flushing, generalized pain, convulsions, chills, fever, headache, phlebitis, anemia, anorexia, and decreased renal function. The most significant of these is the impairment of renal function which occurs in over 80% of persons given Amphotericin B (19). Amphotericin B must be administered parenterally and requires hospitalization and close observation throughout the period of therapy which is usually 6-10 weeks.

Because of the increased incidence of severe Candida infections and the frequency and severity of side effects associated with the treatment of choice, a method of diagnosing systemic candidiasis is badly needed. The physician

needs help in making what is a difficult therapeutic decision because, on the one hand, unnecessary toxic therapy should be avoided but, on the other, a delay in specific anti-fungal therapy in the patient with disseminated disease cannot be afforded. The problem is compounded by the presence of Candida as a commensal and the frequent occurrences of "benign" overgrowth. Differentiation between a superficial and a systemic infection must be made in order to come to a good therapeutic decision.

In too many cases, systemic disease is not diagnosed until after death (20) since the diagnosis can be definitely made only on the basis of histologic studies of infected tissue or autopsy findings. Studies have shown that only 10-44% of cases of disseminated candidiasis documented at autopsy were clinically suspected or diagnosed antemortem (21). Cultures of body fluids, which are a major means of identifying other infecting organisms, are difficult to interpret in the case of candidiasis. Part of this difficulty stems from the ubiquitousness of the organism as a commensal and the frequency of its occurrence as a superficial pathogen. A positive Candida culture from the oral cavity, stool, or vagina is obviously of little diagnostic value since so many individuals harbor the organism at these sites (3). In patients on antibiotics, Candida may proliferate but the mere presence of large numbers of the organism does not necessarily indicate severe infection (20). In a significant number of these cases either with the ad-

ministration of antibiotics or with other predisposing causes, a fungemia is felt to represent a transient contamination of the blood stream rather than seeding from a deep focus of infection (22). Indeed, Candida in blood cultures from patients with intravenous catheters may, on occasion, be eradicated by simple removal of the catheter (23).

Conversely, patients with disseminated candidiasis often have negative blood cultures (24). This may be because of rapid clearing of Candida from the blood by the liver and spleen as has been shown in mice (25). Complement seems to play a role in enhancing the killing of the organisms in other critical target organs such as the kidney (26).

A diagnosis of systemic candidiasis on the basis of clinical data is likewise difficult. The manifestations of dissemination of Candida include chills, high, spiking fever, hypotension, and prostration, none of which can be considered etiologically specific (24). One clue that may indicate the possibility of systemic candidiasis is the presence of numerous cotton-like candidal colonies on the patient's oropharynx. However, since such focal growth is evident in patients without disseminated disease, it is only of inferential value (27). Endophthalmitis is considered to be a pathognomonic clinical sign, but it is not always present (28).

Skin testing has also been investigated as a means of identifying patients with candidiasis, but, since cellular

hypersensitivity to Candida has been demonstrated in 45-80% of persons without candidal infection, skin testing has been largely abandoned as a means of diagnosing candidiasis (3). Another problem is the possibility of anergy in patients with overwhelming infection (29).

Because of these problems with diagnosing systemic candidiasis on the basis of clinical data and culture results, there has been a great deal of interest over the past years in the development of serological tests to be used along with other information to reach a diagnosis. The detection of antibodies in the sera of patients with suspected systemic candidiasis has long been thought of as a potentially rapid and valuable diagnostic method. A number of different techniques of antibody detection have been tried.

Direct slide or tube agglutination of particulate antigen is one of the simplest serodiagnostic tests used in a variety of infectious diseases and its usefulness has been widely investigated with regard to candidiasis (29,30). The result has been that, for several reasons, agglutinin determinations are considered to be of little diagnostic value. Agglutinins have been found in the sera of 20% or more of healthy individuals without evidence of candidal infection, and there is frequent occurrence of cross-agglutination with other microorganisms. In addition, complete absence of agglutinating antibody or insignificant titers have been reported in some 20-25% of patients with systemic

candidiasis (3). Increased sensitivity of agglutination tests has been achieved using latex particles (31) or red blood cells (32) coated with Candida antigen.

The classical complement fixation test has been used in Candida serology but the test has been concluded to be unreliable for clinical diagnostic use and, thus, is not widely utilized today (33).

Precipitation tests have been widely used to detect anti-Candida antibodies. A tube test used by Chew and Theus (34) was shown to be highly sensitive but double diffusion in agar gels (35) has been more widely used because it permits discrimination of the individual precipitin reactions between antibodies and components of the antigen mixture. Counterimmunoelectrophoresis (CIE) (36) is more sensitive than double diffusion as well as more rapid, and has been recommended as a routine test for Candida antibody detection (37). Crossed immunoelectrophoresis has been used to detect and characterize anti-Candida antibodies, providing detailed analyses of the types of precipitins present in patients' sera (38). Immunofluorescence techniques have also been tried, using whole Candida either in the blastospore form (39) or the germ tube form (40), or using Candida polysaccharide antigens engulfed by mouse peritoneal macrophages (41). Many investigators consider elevated or rising titers of immunofluorescent antibody more reliable for systemic candidiasis than high or rising agglutinin titers or the appearance of Candida precipitins

(3,42).

Finally, the most recent technique suggested for detection and quantitation of anti-Candida antibodies has been enzymoimmunoassay (ELISA) (43). The results have been found to correlate well with immunofluorescence antibody titers and ELISA has an advantage in the ease with which numbers of samples can be run.

The wide variety of methods applied to the detection of anti-Candida antibodies is indicative of the problems that remain with the serological diagnosis of candidiasis. Although all of the above-mentioned methods are capable of detecting antibodies, none has achieved a satisfactory degree of specificity, particularly when the differential diagnosis between invasive systemic infection and superficial colonization is the main objective of serological studies. The commensal nature of the organism means that many normal individuals may have antibodies to Candida and many patients with superficial infections demonstrate quite high levels of circulating antibody. In addition, infection with fungi of several different genera may cause production of antibodies which cross-react with Candida albicans (44). On the other hand, patients who have systemic candidiasis may not show antibodies due to immunosuppression or rapid progression to death.

Two general approaches have been tried in an effort to overcome the poor specificity of the currently used techniques of detection of anti-Candida antibodies. One in-

volves quantitation of antibody concentration and is based on the premise that higher titers of antibody may be associated with systemic disease, whereas lower titers are found in superficially infected patients and in the normal population (45). Furthermore, serial samples containing increasing titers might indicate progressive disease, while falling titers might occur with treatment and resolution of the infection.

The second approach involves the refinement of crude extracts presently used as antigens in *Candida serology in* an attempt to produce a preparation that would react specifically with antibodies found in patients with systemic disease. It has been claimed that antibodies to cytoplasmic antigen occur only in systemic candidiasis, whereas antibodies in normal individuals, being the result of superficial colonization, are directed to the mannan of the cell wall (46). Also, it has been shown that the cross-reacting antibodies between other fungi and Candida are directed to the capsular mannan (44). In spite of this, most of the antigens in use at present are crude mixtures containing proteins and polysaccharides, derived from sonically or mechanically disrupted Candida cells (45). For example, the "S antigen" used by Taschdjian and colleagues, obtained by ultrasonication of C. albicans, has been shown to contain cell wall material (45).

In addition, it has been postulated that specificity for systemic Candida infection could be improved by using

antigens from the mycelial phase. As a commensal, Candida exists primarily as yeast, but when causing an infection the mycelial phase predominates (45). Antigenic stimulation from both forms is present in both situations, but there should be a quantitative difference in the antibody response. At the present time, most of the antigens used in serological testing for Candida antibodies are prepared from blastospores, but the use of mycelial antigens for routine diagnostic testing in patients with suspected systemic candidiasis may give more reliable results than are presently obtained with yeast cell antigens (47).

Another approach to improving the rate of successful antemortem diagnosis of systemic candidiasis is through the serological detection of Candida antigens in patients' sera. This would be particularly helpful in patients who are immunosuppressed to such an extent that they are unable to produce antibodies to the infecting organisms. In addition, it is assumed that antigen would be present in patients' sera before the immunologically competent patient has been able to mount an antibody response, and thus, detection of antigenemia would allow earlier diagnosis and earlier treatment. Antigen detection techniques have contributed greatly to the rapid, accurate diagnosis of other fungal and bacterial infections such as Cryptococcal (48) and bacterial meningitis (49), and so it has been seen as a promising approach in the search for a method of diagnosing systemic candidiasis.

Axelsen and Kirkpatrick (50), using crossed immunoelectrophoresis, detected precipitin lines in gel containing sera from a systemic candidiasis patient and antibodies to Candida antigen. There was a reaction of identity with lines in a standard antigen mixture. Weiner and Yount (51) developed a hemagglutination inhibition assay to detect Candida surface antigen, mannan. This assay detected antigen in 30% of patients with invasive candidiasis and in no patients with non-invasive Candida or other systemic infections. Kerkering et al. (52) used CIE and detected polysaccharide antigen in 13 of 48 patients with cultures positive for Candida, 8 of whom were later proven to have systemic candidiasis. The sensitivity in this series remained less than 30%. Other investigators have improved the sensitivity of mannan antigen detection to approach 50% of patients with systemic disease using radioimmunoassay (RIA) (53) and ELISA (54). An RIA developed by Stevens et al. (21) detects soluble cytoplasmic antigen rather than mannan and is said to be positive in approximately 65% of patients with documented disseminated disease. On the other hand, Lehman and Reiss (55) developed antigen detection techniques using immunodiffusion, CIE, and ELISA and were unable to find mannan antigen in any of the human samples they tested including six with proven invasive candidiasis or in the serum of heavily infected and immunosuppressed rabbits. Jones (56) also has questioned the ability of these assays to detect antigen in a significant percentage of patients

with systemic disease.

One postulated reason for the difficulty in detecting circulating Candida antigen in the sera of infected patients is that the antigen may be bound to antibody in the form of circulating immune complexes. Axelsen and Kirkpatrick (50) noted an unusual amount of background staining in the gel of their crossed immunoelectrophoresis and postulated that it might be due to high molecular weight antigen-antibody complexes that did not migrate. Several authors who report success in detecting circulating antigen have found it necessary to treat the sera before testing in a manner that would release antigen from antibody. Segal et al. (57) treated the sera with 0.5 N NaOH while Weiner and Coats-Stephen (53) used acidification with 0.04 M citrate, pH 2.7 and heat treatment.

The presence of circulating immune complexes in other fungal diseases has been suggested. Geha (58) reported C_1q precipitins in the serum of a 13 year old child during acute bronchopulmonary aspergillosis, but the presence of Aspergillus antigen in the complexes was not established. Bullock et al. (59) reported immune complexes in the serum of a patient with disseminated histoplasmosis but again the specific antigen was not demonstrated in the complexes. Yoshinoya et al. (60), studying coccidioidomycosis, not only demonstrated the existence of the circulating immune complexes but also found them to be composed of coccidioidin antigen and specific antibody. These studies provide sup-

port for the idea that antigen circulating in patients with systemic candidiasis might be bound to specific antibody.

Our approach to the diagnosis of systemic candidiasis included investigation into all three phases of the immune response - circulating antigen, immune complexes, and circulating antibody. We began with attempts to develop a more reliable method of diagnosis using antibody detection. Our first approach was to use quantitative immunofluorescence to achieve an estimate of antibody concentration and to determine if higher titers are indeed associated with systemic disease whereas lower titers are found in superficial infection. Our second approach involved the study of the effect of the type of antigen used to detect antibody, be it cytoplasmic protein or cell wall polysaccharide and be it from the yeast or mycelial phase of growth.

We also attempted a number of methods to detect circulating free Candida antigen both in the sera of patients and in the sera of rabbits with experimentally induced Candida endocarditis.

The model for experimental Candida endocarditis was developed by Freedman and Johnson (61) following the establishment of a technique for induction of staphylococcal endocarditis in rabbits (62,63). Al-Doory et al. (64) studied the pathologic changes resulting from induced left-sided fungal endocarditis and found the model to closely resemble endocarditis in man. A number of other investigators (56,65,66) have used the model to study the pathogene-

sis of candidiasis, and we have used it as a source of materials on which to base development of antigen detection methods.

Our lack of success with these methods led us to investigate the possibility that the antigen might be bound to antibody and thus, that circulating immune complexes containing Candida antigen and specific antibody might be found in sera. We thus performed screening tests for circulating immune complexes on a number of patients with candidiasis and attempted to isolate and characterize the complexes found in some of them.

Materials and Methods

I. Patients and Normal donors

In the development of the quantitative immunofluorescence assay, serum samples from nine patients suspected of having systemic candidiasis on the basis of suggestive clinical features and positive blood cultures of C. albicans were used along with samples from 43 normal donors with no clinical symptoms of Candida infection. The normal donors were divided into two groups, according to the presence or absence of precipitating anti-Candida antibodies in their sera.

In the studies using purified antigens, two different series of patients were used. Fifty-seven sera were included in Series I and categorized as follows: Group I consisted of 17 sera obtained from patients who had positive cultures for Candida species from non-cutaneous sites. These were divided into Group Ia, including nine patients with clinical evidence of systemic candidiasis and positive reaction when tested against a crude extract of C. albicans yeast by double immunodiffusion , and Group Ib, including eight patients with no clinical evidence of systemic candidiasis and negative reaction in the double immunodiffusion test. Group II consisted of ten sera known

to contain antibodies against Histoplasma and Cryptococcus. Group III was made up of sera from 30 healthy individuals without any sign of disease. It is probable that some of the persons in this group suffered from subclinical mucosal candidiasis, since this condition is relatively frequent.

Series II consisted of 44 patients with various forms of candidiasis and ten normal controls. In this series, we established strict criteria for a diagnosis of disseminated candidiasis: 1) two or more positive blood cultures for Candida obtained on different samplings with at least one positive in the absence of or after removal of any potential intravenous catheter source; (2) evidence of Candida endophthalmitis, with or without positive blood cultures; 3) positive culture for Candida from cerebrospinal, pleural, or peritoneal fluid; 4) positive culture of peritoneal fluid in the absence of recent abdominal surgery, or positive peritoneal fluid and blood cultures in the presence of recent abdominal surgery; 5) histologic or culture evidence of Candida at multiple tissue sites other than normal skin and mucous membranes. All of the patients in Groups I and II of Series II fit one or more of the criteria described above. The seven patients in Group I had candidemia with multiple positive cultures and definite evidence of a deep focus of infection leading to a proven diagnosis of systemic candidiasis. Group II consisted of five patients with candidemia with multiple positive cultures and clinical evidence leading to a probable diagnosis of

systemic candidiasis. Group III consisted of three patients with clinical evidence of systemic candidiasis but negative blood cultures. Group IV consisted of three patients with bladder or other non-invasive local infections. These patients had: 1) histologic or culture evidence of Candida at a single tissue site other than normal skin or mucous membranes; 2) ≧ 100,000 colonies of Candida isolated from the urine as the sole organism on at least two different occasions with associated pyuria (≧ 10 WBC/HPF on spun urine).

Group V consisted of 17 patients with vaginal candidiasis. These patients met three criteria: 1) symptomatic vaginitis (burning, itching, and/or leukorrhea); 2) characteristic findings of vaginitis on pelvic examination; and 3) the finding of budding yeast with pseudohyphae on microscopic examination of 20% KOH wet preparations of high vaginal secretions or plaque scrapings. All patients who were cultured had growth of C. albicans from the secretions.

Group VI consisted of nine patients with cutaneous or mucosal candidiasis who had Candida isolated from the normal skin, stool, or mouth.

II. Animal Studies

Production of Candida endocarditis in rabbits

Endocarditis caused by Candida albicans was induced in New Zealand white rabbits weighing 2 kg accord-

ing to the method of Sande et al. (66). The right carotid artery was exposed and ligated and a sterile 19 gauge polyethylene catheter was inserted across the aortic valve. The catheter was then clamped and the skin was closed over it. Candida infection was established by the intravenous injection 48 hours later of 10^6 Candida cells. The organisms had been grown on Sabouraud's dextrose agar for 48 hours and washed from the slants with sterile saline. After three washes with sterile saline, the cells were counted in a hemocytometer and adjusted to a concentration of 2×10^6/ml. 0.5 ml was injected into the marginal ear vein of each rabbit. As controls, some rabbits were catheterized but not infected while others were infected without previous catheterization.

Collection of specimens

Rabbits were bled prior to infection and at frequent intervals after infection, generally four times during the first week and twice each week thereafter. Sera was collected and stored at -20°C, and at each bleed a blood culture was done by innoculating 1 ml of blood into 10 ml of sterile brain heart infusion agar. The cultures were incubated at 37°C with constant gentle agitation and subcultured at one day and at one week.

At the time of death from disease or after sacrifice approximately four weeks post-infection, the animals were autopsied. Tissue samples were taken from heart, kidney, and other organs with gross signs of infec-

tion for culture and for pathologic studies. The tissue for culture was placed into sterile bottles of brain heart infusion agar and treated as were the blood cultures. The tissue for pathologic studies was formalin fixed, embedded in paraffin, sectioned and stained with PAS.

Sera were tested for antibody and antigen by counterimmunoelectrophoresis and by quantitative immunofluorescence.

III. Antibody Detection Methods

Immunodiffusion

Ouchterlony double immunodiffusion (67) was carried out in 1% Difco (Detroit, Mich.) Noble agar prepared with 0.3 M phosphate buffer, pH 8.0 (68).

Counterimmunoelectrophoresis (CIE)

CIE studies were carried out on 8 by 5 cm glass plates coated with 8 ml of 1% LSA agarose (Litex, Accurate Chemical and Scientific Corp., Westbury, N. Y.) prepared in 0.5 M barbital buffer, pH 8.6, containing 1% (w/v) dextran T70 (Pharmacia Fine Chemicals, Uppsala, Sweden). The antigen and antibody wells had a diameter of 3 mm, and their closer edges were 4 mm apart. Each well was filled with 10 µl of either antigen or antibody. The electrophoretic separation was carried out at 150 V for 90 minutes. After completion of the run, the plates were read on a dark field illuminator, washed twice in saline,

once in distilled water, dried, and stained with amido black. The readings were repeated after staining.

Quantitative immunofluorescence (QIF)

Uncoated IDT StiQs were obtained from International Diagnostic Technology (Santa Clara, California). These StiQs consist of a plastic handle with an immunoabsorbent disc affixed to a rounded tip which can be inserted into tubes containing test solutions and later into the fluorometer. The IDT StiQs were spotted with 25 µl of antigen and allowed to dry at room temperature overnight. After drying, the StiQs were washed in 600 µl of Tris-buffered saline, pH 8.1, containing 0.2% Tween 20, for 30 minutes at room temperature with constant shaking. After this wash, the StiQs were again dried at room temperature and used immediately or stored in a sealed box at 4°C until needed.

The protocol for determination of anti-*Candida* antibodies in serum samples was as follows: (a) serum was diluted in Tris-buffered saline, pH 8.1; (b) StiQs were incubated with 600 µl of each serum dilution for 30 minutes at room temperature, with constant gentle shaking; (c) after the incubation, the StiQs were washed for 10 minutes in 600 µl Tris-buffered saline at room temperature with constant shaking; (d) after the wash, the StiQs were incubated with 600 µl of fluorescein-labeled antiserum to human immunoglobulins (Burroughs-Wellcome, Research

Triangle Park, Durham, N. C.), with constant shaking at room temperature, and (e) this was followed by a 10 minute wash in 600 µl of Tris-buffered saline as described above. After the last wash, the StiQs were read in the fluorometer. As a control for specificity of staining, antigen-coated StiQs were incubated with 600 µl of Tris-buffered saline instead of human serum and processed in an identical fashion.

When determination of antibody class was the aim of the assay, monospecific antiserum to human IgG or IgA (Burroughs-Wellcome, Research Triangle Park, Durham, N. C.) was used in the final incubation step. To assure that the monospecific antisera to human IgG and IgA were of comparable antibody titers, StiQ samplers were coated with 25 µl of 10 µg/ml solutions of purified IgG or IgA and were incubated in 1/100 dilutions of monospecific antisera. Means of readings on the fluorometer of six StiQs with each antigen were compared using the Student's t-test and no significant difference was found at the level of $p < 0.05$.

The FIAX fluorometer (International Diagnostic Technology, Santa Clara, Calif.) is a computerized instrument that allows quantitative readings of fluorescence. Light from a highly stabilized tungsten filament source passes through a narrow band interference filter whose optical passband is centered at 540 nm, which passes only the fluorescent signal produced by the sample. Light is focused onto the photocathode of a photomultiplier tube, which

converts the sample signals to electrical signals. The electrical signals are converted to a three-digit reading and displayed on a digital panel.

Indirect Immunofluorescence (IIF)

Candida albicans type A strain B385 were cultured in brain heart infusion agar (Difco Laboratories, Detroit, Mich.) pH 7.4, at 25°C (69) for 48 hours, then killed by the addition of formalin to a final concentration of 0.5%. Cells were harvested by centrifugation, washed with formalinized saline, and finally suspended at 1.6 x 10^6/ml in phosphate-buffered saline, pH 7.4. One drop of this suspension was used to make a smear which was air-dried and heat-fixed. A 1 cm diameter circle was marked on each slide. 25 µl of serum diluted 1:5 in PBS was placed over the cells in the circle and the slide was allowed to stand in a moist chamber for 30 minutes at room temperature. Slides were rinsed with three changes of PBS for five minutes each. One drop of fluorescein-labeled anti-human IgG or IgA, diluted 1:20 in PBS was placed over the cells within the circle and allowed to stand in a moist chamber for 30 minutes. Slides were washed as before, mounted in buffered glycerol, and viewed under a Leitz fluorescent microscope.

IV. Preparation of antigens for use in antibody detection

Antigen for immunodiffusion

Whole cell preparations were made according

to Sweet and Kaufman (70). Cells were mechanically disrupted in a hydraulic press and centrifuged at 10,000 x g for 30 minutes. The final supernatant fluid, concentrated and adjusted to 5 mg/ml protein concentration, was used as the antigen in immunodiffusion studies.

Antigens for CIE and QIF

A commercially prepared extract of Candida albicans, preserved in 50% glycerol, was obtained from Hollister Stier (Division of Cutter Laboratories, Berkeley, Calif.). For use in CIE, the antigen was diluted 1:50 in 0.15 M phosphate-buffered saline, pH 7.2. For use in QIF the antigen was dialyzed against three changes of 0.05 M Tris-buffered saline, pH 8.1. The protein concentration of the dialyzed antigen was determined by the Bio-Rad protein assay method (Bio-Rad Laboratories, Richmand, Calif.), and, if needed, the protein was concentrated by negative pressure ultrafiltration. In the development of the QIF assay, the antigen was used at two concentrations, 0.15 mg/ml and 0.35 mg/ml. Later, the standard concentration used was 0.15 mg/ml.

Purified mycelial antigens were prepared as follows. A culture of C. albicans type A B385 was maintained on Sabouraud's dextrose agar (Difco Laboratories, Detroit, Mich.). Cells from this stock culture were inoculated into 100 ml of the amino acid containing medium formulated by Lee et al. (71) in a 250 ml Ehrlenmeyer

flask and rotated at 200 rpm at 15°C. When this initial culture reached stationary phase, at a blastospore concentration of approximately 2 x 10^8/ml, the culture was diluted 1:15 in fresh medium (pH 6.5), bringing the total volume to two liters, and then incubated at 37°C in a rotating incubator (200 rpm) to induce transition to the mycelial phase (72). The culture was harvested by centrifugation (3000 x g) and washed three times with phosphate-buffered saline, pH 7.2. The washed cells were disrupted by three passages through a French pressure cell (American Instrument Company, Silver Springs, Maryland), frozen at -20°C, sonicated, and passed by pressure two more times, resulting in complete destruction of the cells (73). The resulting homogenate was centrifuged in three stages at 4°C - 10,000 x g for 20 minutes, 45,000 x g for 30 minutes, and 100,000 x g for one hour - to produce a clear supernatant (74).

The crude extract was concentrated and dialyzed for 24 hours against several changes of buffer consisting of 0.01 M Tris amino-methane-buffered saline, pH 7.2, containing 10^{-3}M $CaCl_2$ and 10^{-3}M $MnCl_2$ (73). After filtration through a 0.45 μm polycarbonate membrane (Nuclepore, Pleasantown, Calif.), 12 ml of the crude extract was applied to a Sepharose-ConA (Pharmacia Fine Chemicals, Uppsala, Sweden) (25 ml bed volume) column. This column had previously been equilibrated with the same buffer. Chromatography was carried out at room temperature.

Unbound material was eluted with the equilibrating buffer. The material bound to the ConA was eluted with 0.2 M methyl mannoside in water (74). The bound and unbound fractions were concentrated and dialyzed against phosphate-buffered saline, pH 7.2. The total protein concentration and carbohydrate concentration were determined in both fractions. The unbound fraction is hereafter called mycelial cytoplasmic antigen; the bound fraction, mycelial polysaccharide antigen.

Purified yeast antigens were prepared as follows. Cells from the stock culture of C. albicans A B385 were streaked onto slants of Sabouraud's agar and incubated for 18 hours at room temperature. After microscopic determination that the organisms were growing in the yeast phase, they were washed from the slants into 300 ml of brain heart infusion agar in 500 ml bottles and rotated for 48 hours at 25°C (75). 1.5 ml of formalin was added to each bottle and incubation continued at 25°C for six hours. Cultures were harvested by centrifugation at 3000 x g and washed three times with 0.5% formalinized saline. The final pellet was suspended at 25% (v/v) in formalinized saline. Purification was continued as for mycelial cells resulting in a yeast cytoplasmic antigen preparation and a yeast polysaccharide antigen preparation.

An aliquot of the Hollister-Stier extract of Candida albicans was also purified. Fifteen ml of the extract was dialyzed for 72 hours against daily changes of

distilled water. The antigen was concentrated and treated in the same fashion as the crude mycelial extract and then chromatographed on a Sepharose-ConA column.

The protein concentration in the various antigenic preparations was determined by the Coomasie blue dye-binding technique (76) (Bio-Rad Laboratories, Richmond, Calif.), using bovine gamma globulin as a calibration standard. The phenol-sulfuric acid colorimetric method was used for total carbohydrate analysis (77). A standard curve based on a mixture of sugars was used for the quantitation of the carbohydrate.

For CIE the cytoplasmic antigen preparations were used at a protein concentration of 1 mg/ml and the polysaccharide antigen at a carbohydrate concentration of 170 µg/ml. For QIF the cytoplasmic antigen was used at a protein concentration of 0.25 mg/ml and the polysaccharide antigen at a carbohydrate concentration of 90 µg/ml.

V. Carbohydrate Analysis of C. albicans antigens

Carbohydrates in the antigen preparations were further analyzed by gas-liquid chromatography. An aliquot of each preparation, adjusted to contain 100 µg of sugar, was dried under a stream of nitrogen, then heated for 16 hours at 65°C with 1 ml of 500 mM HCl in anhydrous methanol. The samples were again dried under a stream of nitrogen, and N-acetylation of the free amino groups was achieved by suspending the residue in a mixture of 0.1 ml acetic anhy-

dride. The samples were immediately evaporated to dryness and the simultaneous O-acetylation was reversed by refluxing in 1 ml of 500 mM HCl diluted in anhydrous methanol for one hour (78).

The constituent carbohydrates were converted to o-trimethyl-silyl ethers (o-TMS ethers) by treatment with Tri-Sil (Pierce Chemical Co., Rockford, Ill.) for 15 minutes at 65°C (79). The samples were then dried, and the o-TMS ethers dissolved in 0.5 ml hexane solution were injected into a Varian 4000 dual column gas-liquid chromatograph fitted with an OV-17 column. The initial temperature was 90°C with a 1 minute hold, increasing 10° per minute thereafter until reaching an upper limit of 250°C. This temperature was held for 10 minutes. Nitrogen was used as a carrier gas, at a flow rate of 37.5 ml/minute. The separations were calibrated with o-TMS ethers of mannose and glucose.

VI. Antigen Detection Methods

Counterimmunoelectrophoresis

CIE studies for antigen detection were carried out as were those for antibody detection.

Two different types of antibody were used to detect antigen. A commercial preparation of rabbit immunoglobulins to Candida albicans was obtained from DAKO-immunoglobulins (Copenhagen, Denmark). The antisera was further purified by chromatography on a DEAE-Sephacel

column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.01 M Tris-HCl, pH 7.0 (80). The column was eluted with 0.05 M Tris-HCl, pH 7.0 to obtain Peak I and then with the same buffer containing 0.25 M NaCl to obtain a second peak. A final wash was done with 0.05 M Tris-HCl, pH 7.0 with 1 M NaCl. The peaks were concentrated by ultrafiltration and checked for antibody activity by CIE and for immunoglobulin purity by immunoelectrophoresis (81). Protein concentration was determined by the Bio-Rad protein assay method. CIE showed that the majority of the antibody activity was in Peak I, and IEP showed that Peak I was pure IgG.

Antibody was also obtained from the sera of two patients (VL and LS) with a high titer of anti-Candida antibodies. These sera also were applied to DEAE-Sephacel equilibrated with 0.01 M Tris-HCl, pH 7.0, and the peak containing pure IgG with maximal antibody activity was eluted with the equilibrating buffer.

The limits of antigen detection by both the purified commercial rabbit immunoglobulin and the purified human antisera were determined by CIE. The rabbit antibody at 1 mg/ml detected 500 ng/ml of Candida antigen. The antibody purified from VL sera at a concentration of 0.5 mg/ml detected 200 ng/ml of antigen. The antibody purified from LS sera at a concentration of 2 mg/ml detected 200 ng/ ml of antigen.

Quantitative Immunofluorescence Inhibition Assay

Uncoated IDT StiQs were spotted with 25 µl of *Candida* antigen, a yeast extract with a protein concentration of 50 µg/ml and a carbohydrate concentration of 50 µg/ml. After being allowed to dry at room temperature overnight, the StiQs were washed in 600 µl of the assay buffer, Tris-buffered saline, pH 8.1 containing 0.2% Tween 20. In the assay, StiQs were incubated for 30 minutes in 600 µl of one of the following: rabbit anti-*Candida* immunoglobulins (DAKO) diluted 1:200 in assay buffer as the 100% positive control; a preincubated mixture of equal volumes of rabbit anti-*Candida* diluted 1:100 and various concentrations of *Candida* antigen to establish a standard curve; a preincubated mixture of equal volumes of rabbit anti-*Candida* 1:100 and sera to be tested. Following this incubation, the StiQs were washed in 600 µl of assay buffer for 10 minutes at room temperature with constant shaking. Then, StiQs were incubated with 600 µl of fluorescein-labeled sheep anti-rabbit immunoglobulins (Burroughs-Wellcome, Research Triangle Park, Durham, N. C.) diluted 1:100 in assay buffer for 30 minutes at room temperature. After a final ten minute wash in assay buffer, the StiQs were read in the FIAX fluorometer.

ELISA Inhibition Assay

A modification of the method of Segal et al. (57) was used. Cuvettes supplied as the solid phase

for the Gilford EIA Automatic Analyzer System PR-50 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) were coated with Candida yeast extract antigen. 100 µl of the antigen diluted to a concentration of 2 µg/ml in 0.1 M carbonate buffer, pH 9.6 was incubated in each test well of the cuvette overnight at 4°C. Control wells were incubated with carbonate buffer without antigen. After this incubation, the cuvettes were washed four times on the processor of the Gilford EIA automatic analyzer, using as assay buffer, 0.15 M phosphate-buffered saline, pH 7.2 containing 0.05% Tween 20. In the next step, 100 µl of one of the following was added to a cuvette well and incubated at 37°C for 30 minutes: PBS with Tween 20 as a control; rabbit anti-Candida antibodies (DAKO) diluted 1:1000 in the assay buffer as the 100% positive sample; a preincubated mixture of equal parts of rabbit anti-Candida diluted 1:500 and Candida antigen at various concentrations to establish a standard curve; or a preincubated mixture of equal parts of rabbit anti-Candida immunoglobulins 1:500 and sera to be tested. After this incubation, the cuvettes were washed seven times with the assay buffer. Then, 100 µl of horseradish peroxidase-labeled swine anti-rabbit IgG (Cappel Laboratories, Downington, Pa.) diluted 1:1000 in assay buffer was added to each well for a one hour incubation at 37°C. Again, the cuvettes were washed seven times with the assay buffer. Then, 200 µl of freshly made working substrate solution was added to each

well. The working substrate solution consisted of 1% stock substrate and 0.3% hydrogen peroxide (Eckerd Corporation, Clearwater, Fla.) in 0.05 M citrate phosphate buffer, pH 5.0. The stock substrate was made up weekly as 0.001% orthophenylenediamine (Sigma Chemical Company, St. Louis, Mo.) in methanol. After a 45 minute incubation in the dark at room temperature, 200 µl of 4 N sulfuric acid was added to each well to stop the reaction. The color reaction was read by the spectrophotometer incorporated into the Gilford system at a wavelength of 490, and the measured absorbance value with a range of 0 to 2.0 was printed out by a thermal printer.

Sera to be tested in both types of inhibition assay were used either untreated or treated by the method of Segal et al. (57) to remove any bound anti-Candida antibodies. In this method, one volume of 3 N NaOH was added to 5 volumes of sera and the mixture was incubated for 2 hours at 56°C. After centrifugation at 2000 x g for 5 minutes, the supernatant was dialyzed against PBS before testing in the assay.

VII. Immune Complex Studies

Immune Complex Screening Techniques

Direct nephelometry

A modification of the method previously described by Virella et al. (82) was used. The method consists of determining the effects of polyethylene glycol

6000 (Fisher, Fairlawn, N. J.) on the immune complexes in the sample, assuming that an increase in light scattering reflects the specific aggregation of soluble immune complexes. Light scattering is measured in a Hyland PDQ Nephelometer (Hyland Diagnostics, Deerfield, Ill.), with the sensitivity set at 3 and 7 (course and fine) and a final adjustment to obtain a reading of 170 to 190 using a reference latex suspension (0.010 ml of a suspension of polystyrene latex particles, 0.035 μm in diameter (Dow Diagnostics, Indianapolis, Ind.) in 10 ml of 3% (w/v) polyethylene glycol 6000 solution in 154 mM NaCl. The sample values are recorded as a percentage of the latex suspension reading, after the sample blank values have been obtained.

PEG-IgG and PEG-C_4 Tests

These are modifications of the original procedures of Digeon et al. (83). The samples to be tested are incubated overnight at 4°C with 3% (w/v) polyethylene glycol 6000 to 0.01 M borate-buffered saline, pH 8.4. The resulting precipitates are washed twice in 3% polyethylene glycol 6000 at 4°C and resuspended in 0.4% Tween 20 in borate buffer and incubated at 37°C for 30 minutes. The IgG and C_4 are assayed nephelometrically in both the original serum and the resuspended precipitates. The results are expressed as a ratio between IgG or C_4 in the precipitate and the corresponding value in the serum.

^{125}I C_1q Binding Assay

A modification of the method of Zubler and Lambert (84) was used. C_1q was isolated by the method of Volanakis and Stroud (85) and labeled by the method of McConahey and Dixon (86). Purity was determined by double immunodiffusion and immunoelectrophoresis. Biological activity was assessed by measuring the ability of the C_1q to agglutinate IgG-coated latex (Rheuma-Wellcotest, Wellcome Diagnostics Research Triangle Park, Durham, N. C.). The results of the binding assay were expressed as:

$$\%C_1q \text{ binding} = \frac{\text{sample value}}{\text{x of controls}} \times 100$$

IC score

The results of the immune complex screening tests were used individually and were compiled by the method of Kilpatrick and Virella (87) into an IC score. This score was derived in the following manner: the mean value of the normal control population for a given test was subtracted from the value for a given sample in the same assay, and the resulting difference was divided by the standard deviation of the values obtained with the normal control. The mean of the corrected scores for the different assays was then recorded as the IC score.

Isolation of Soluble Immune Complexes

The method described by Virella et al. (88) was employed for the isolation of soluble immune complexes. Briefly, 1 ml of 20% polyethylene glycol 6000 in

borate buffer was added slowly to 3 ml of serum to be examined. The mixture was allowed to stand at room temperature for one hour and at 4°C for two hours. This mixture was then centrifuged at 35,000 x g for 30 minutes and the precipitate was suspended in 500 mM potassium phosphate buffer, pH 7.5 and dialyzed against three changes of 0.1 M phosphate-buffered saline, pH 7.3. The sample was applied to a 5.5 by 34 cm column of ACA -34 (LKB Instruments, Inc., Rockville, Maryland) equilibrated with PBS for separation of fractions by molecular weight. The higher molecular weight fractions were pooled and concentrated by negative pressure ultrafiltration and applied to a 1.5 by 10 cm column of Sepharose 4B - Protein A (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was eluted with PBS with the elution monitored at 280 nm. After the elution of the unbound protein, when the eluate had an optical density near 0, 0.2 M acetate buffer, pH 3.8 was used to elute the bound immune complexes.

For the final dissociation of antigen and antibody, the protein eluted with the acid buffer from the protein A affinity chromatography column was submitted to a final fractionation at pH 3.8 on a 1.5 by 60 cm column of ACA-34.

Characterization of Isolated Immune Complexes

Immunochemical characterization of the proteins separated at different stages of the isolation proto-

col were carried out using double immunodiffusion, performed in 1% Difco Noble agar in 0.02 M potassium phosphate buffer, pH 8.0. Anti-sera used in these studies was as follows: goat anti-human IgG heavy chain (Antibodies, Inc., Davis, Calif.); sheep anti-human IgM, heavy chain specific (Cappel Laboratories, Cochranville, Pa.); goat anti-human IgA, heavy chain specific (Cappel Laboratories); goat anti-human C_1q (Atlantic Antibodies, Scarborough, Maine); goat anti-human C_3 (Cappel Laboratories); and goat anti-human C_4 (Atlantic Antibodies).

Fractions from different stages of the isolation procedure were tested for the presence of anti-Candida antibodies by counterimmunoelectrophoresis and by quantitative immunofluorescence. Candida antigen was detected by counterimmunoelectrophoresis and by the quantitative immunofluorescence inhibition assay.

VIII. Statistical Analysis

Statistical analysis of the distribution of antibody levels as determined by quantitative immunofluorescence indicated skewness not consistent with a normal distribution of values. Therefore, comparison of antibody levels in patient and normal groups was accomplished using non-parametric techniques. Wilcoxon's two sample test as extended by Mann and Whitney to deal with unequal-sized samples was used in the studies on antibody detection (89).

In the immune complex studies data was also anal-

yzed by the Mann-Whitney test to determine the level of significance of the differences between normal and patient populations.

Results

I. Development of the QIF Assay

Our attempts to improve the specificity of the antibody detection methods of diagnosis of systemic candidiasis through quantitation of antibody concentration resulted in the development of a new assay using quantitative immunofluorescence. Counterimmunoelectrophoresis was used as the reference technique in the development of the new assay.

In our initial experiments, we determined the optimal concentrations of antigen and fluorescent antiserum for the assay. Commercial Hollister-Stier antigen was used at this stage of the investigation. Two sera were used, one strongly positive and the other negative by CIE for anti-*Candida* antibodies, both at a dilution of 1:20. The StiQs were spotted with *C. albicans* antigen at two different concentrations, 0.15 and 0.35 mg/ml. The fluorescein-labeled antiserum to human immunoglobulins was used at three different dilutions, 1:20, 1:50 and 1:100. The results of these experiments (Table 1) showed good differentiation between the positive and negative samples at all combinations of antigen and antibody tested. We observed no fluorescent staining when antigen-spotted StiQs were in-

cubated with Tris-buffered saline instead of human serum. In all subsequent experiments, the antigen was used at a concentration of 0.15 mg/ml and the fluorescent antiserum was diluted 1:100.

The objective of the next series of experiments was to determine whether a correlation could be established between different concentrations of a positive serum and the readings obtained with the fluorometer, keeping the concentrations of antigen and fluorescent-labeled antiserum constant. Figure 1 illustrates the correlation between the intensity of fluorescence and the dilution of positive serum. The range of dilutions 1:40 to 1:1280 was chosen to encompass the interval in which antibody concentrations of a majority of unknown sera fell, and to reach the lower limit of sensitivity of the assay. The lowest dilution of the positive serum (1:40) was considered as 100%, and each following dilution was expressed as a percentage of this maximum. The correlation obtained (Figure 1) permitted calibration of the assay.

When unknowns were assayed, each sample was diluted 1:40 and the samples were run in duplicate. The fluorescence readings obtained in the duplicate assays were converted into percentages of the positive control. The results of our assays of an initial group of unknown samples and the results of CIE assays of the same samples are shown in Table 2. The sera were grouped into three categories according to whether they were obtained from patients

with clinical and cultural evidence of systemic candidiasis (Group I) or from normal controls who were positive (Group II) or negative (Group III) for precipitating antibodies to C. albicans as detected by CIE. Statistical analysis of the groups by the Mann-Whitney test showed that antibody levels in the Group I subjects were significantly different from the levels of normals in Groups II and III ($p < 0.01$). Similarly, significant differences ($p < 0.01$) were found in comparisons of Groups II and III.

Finally, we tested the reproducibility of the assay by repeating the determinations of five sera. Five tests of each sample were run in one experiment to determine within run reproducibility. The same samples were run in duplicate on five other occasions; the results of these repeated determinations are shown in Table 3.

II. Use of Purified Candida Antigens

Once this quantitative assay for anti-Candida antibodies was developed, we redirected our approach to improving the serodiagnosis of Candida toward the area of refinement of antigens used to detect the antibodies.

Analysis of the Antigens

The preparation of mycelial and yeast extracts as described in Materials and Methods provides a mixture of antigens in which proteins predominate over carbohydrates (Table 4). Affinity chromatography on ConA-

TABLE 1

STUDY OF THE EFFECTS OF VARIATIONS IN ANTIGEN AND FLUORESCEIN-LABELED ANTIBODY CONCENTRATIONS ON THE INTENSITY OF FLUORESCENCE OBTAINED WITH SERUM FROM A PATIENT WITH SYSTEMIC CANDIDIASIS AND WITH SERUM FROM AN ASYMPTOMATIC CONTROL

Antiserum	Antigen at 0.15 mg/ml		Antigen at 0.35 mg/ml	
	Patient[a]	Control[a]	Patient[a]	Control[a]
FITC-anti-Ig 1/20	184	19	195	29
FITC-anti-Ig 1/50	200	23	200	31
FITC-anti-Ig 1/100	164	16	148	22

[a]Both sera diluted 1/20. The fluorescence intensity measurements are expressed in arbitrary units, as calculated by the FIAX fluorometer.

Figure 1

Semi-log plot of fluorescence intensity vs. antibody concentration obtained with a series of doubling dilutions (1/40 to 1/1280) of a serum containing anti-C. albicans precipitates at a titer of 40 by CIE. The intensity of fluorescence is expressed in the units given by the fluorometer readouts. The antibody concentrations are expressed as percentages of the lowest dilution of the positive serum (designated as 100%).

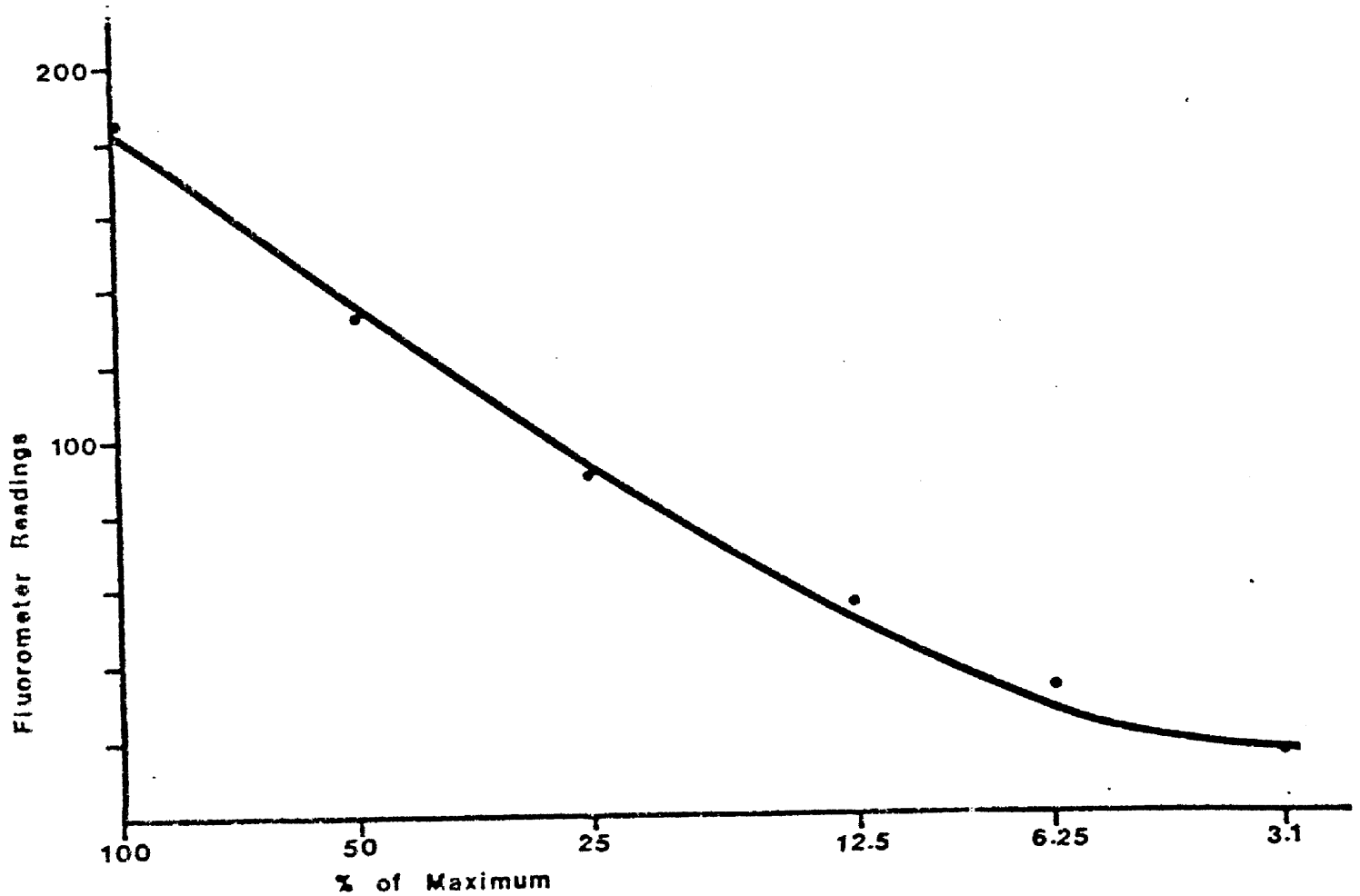


TABLE 2

RESULTS OF QUANTITATIVE IMMUNOFLUORESCENCE AND COUNTERIMMUNOELECTROPHORESIS DETERMINATIONS OF ANTI-*CANDIDA* ANTIBODIES

Group I[a]			Group II[b]			Group III[c]		
Case no.	QIF (%)	CIE	Case no.	QIF (%)	CIE	Case no.	QIF (%)	CIE
1	>100	+	10	22.1	+	28	8.2	-
2	100	+	11	11.4	+	29	7.8	-
3	31	+	12	9.4	+	30	6.6	-
4	22	+	13	9.0	+	31	4.2	-
5	18	+	14	8.6	+	32	4.0	-
6	12	+	15	8.4	+	33	3.8	-
7	11.4	+	16	8.4	+	34	3.4	-
8	8.3	+	17	8.4	+	35	3.0	-
9	5.7	+	18	7.9	+	36	3.0	-
Mean	26.0	+	19	6.3	+	37	2.8	-
±S.D.	±30.9	+	20	5.7	+	38	2.7	-
			21	5.7	+	39	1.9	-
			22	5.1	+	40	1.8	-
			23	5.0	+	41	0.6	-
			24	5.0	+	42	0	-
			25	3.5	+	43	0	-
			26	3.0	+	44	0	-
			27	0	+	45	0	-
			Mean	7.5%		46	0	-
			±S.D.	±4.5%		47	0	-
						48	0	-
						49	0	-
						50	0	-
						51	0	-
						Mean	2.2%	
						±S.D.	±2.6%	

[a]Group I, patients with systemic candidiasis

[b]Group II, normals with positive precipitin test for anti-*Candida* antibodies.

[c]Group III, normals with negative precipitin test for anti-*Candida* antibodies.

TABLE 3

WITHIN-RUN AND DAY-TO-DAY PRECISION OF THE QUANTITATIVE IMMUNOFLUORESCENCE ASSAY FOR ANTI-CANDIDA ANTIBODIES

Within-run (n = 5)		Day-to-Day (n = 5)	
Mean QIF%	CV%	Mean QIF%	CV%
2.98	9.4	4.36	30.96
3.04	8.55	4.01	19.20
5.52	13.41	8.10	40.74
18.06	4.21	20.21	10.49
11.76	18.45	18.63	14.65
Mean	10.80	Mean	23.21

Sepharose resulted in the separation of two fractions, one containing the unabsorbed material washed out with the equilibrating buffer (Fraction I) and the other (Fraction II) containing polysaccharides bound to the insolubilized ConA and specifically eluted with alpha-methyl mannoside. Chemical analysis of both fractions showed that Fraction II was practically devoid of protein.

The commercially-prepared Hollister-Stier antigen was found to be rich in carbohydrates; fractionation by affinity chromatography on ConA-Sepharose also resulted in the separation of two fractions, an unbound Fraction I, rich in proteins and a bound Fraction II, rich in carbohydrates.

The nature of the carbohydrates present in the crude antigen preparations and their respective fractions was studied by gas chromatography. These studies allowed the identification of glucose and mannose in all antigenic preparations containing detectable sugar. Representative profiles obtained in these studies are shown in Figure 2. Basically, these results show that mannan is present as a contaminant in crude extracts and can be removed by affinity chromatography using Sepharose-ConA, as previously shown by Longbottom et al. (73).

Comparison of Reactions with the Various Antigens Using CIE

Initial tests of sera from patients in

TABLE 4

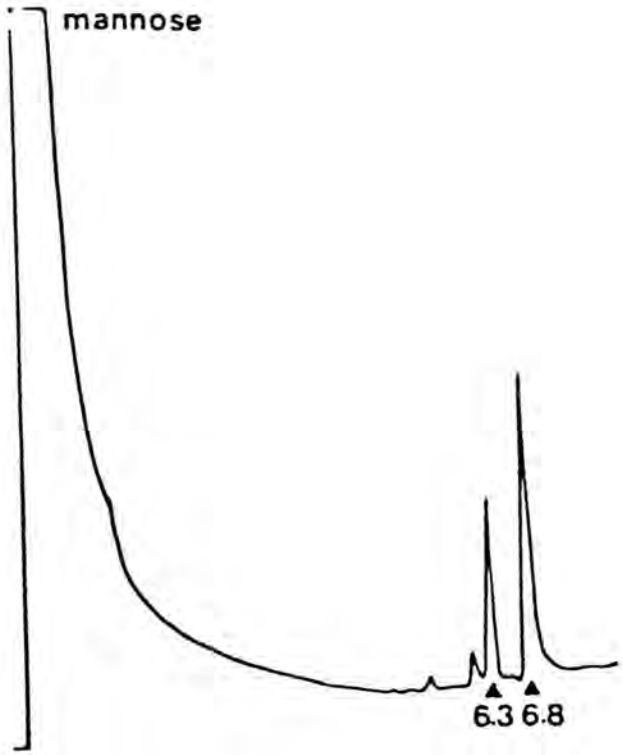
CHEMICAL ANALYSIS OF THE CRUDE MYCELIAL AND YEAST EXTRACTS AND OF THE HOLLISTER-STIER ANTIGEN AND THEIR RESPECTIVE FRACTIONS OBTAINED BY AFFINITY CHROMATOGRAPHY ON CONA-SEPHAROSE

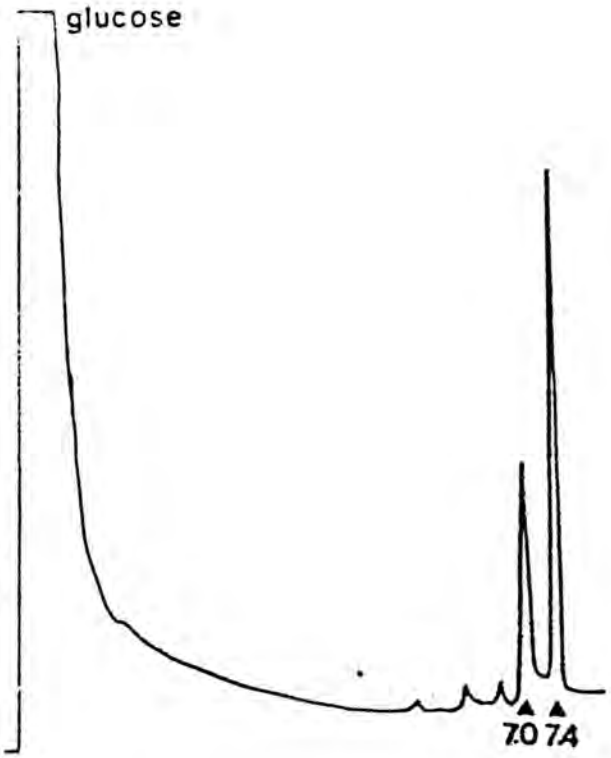
	Mycelial ag			Yeast ag			Hollister-Stier		
	Crude	Frac I	Frac II	Crude	Frac I	Frac II	Crude	Frac I	Frac II
Protein conc. (mg/ml)	2	1	ND	2.2	2.7	ND	0.3	0.1	0.1
Carbohydrate conc. (mg/ml)	0.75	ND	0.17	2.65	ND	0.18	1.2	0.075	0.39

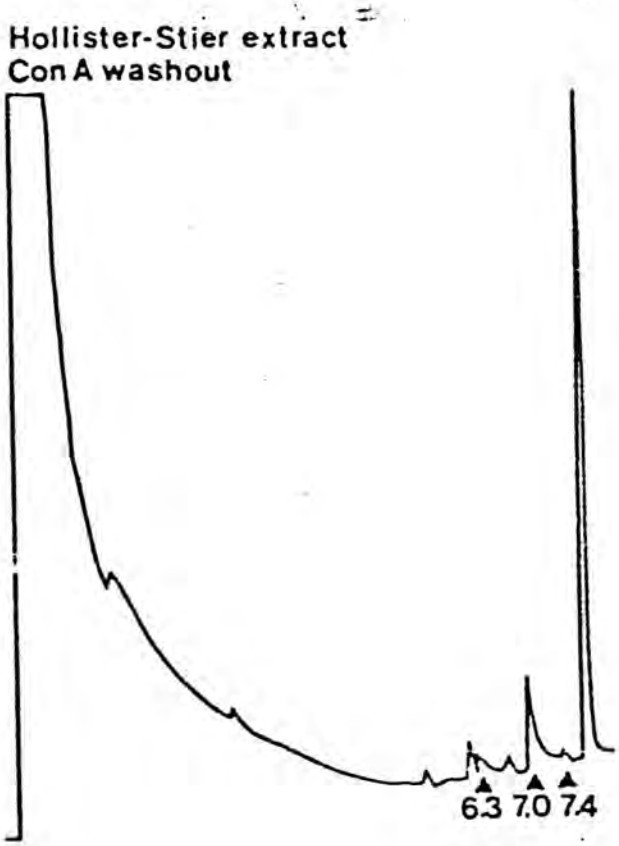
ND - not detectable

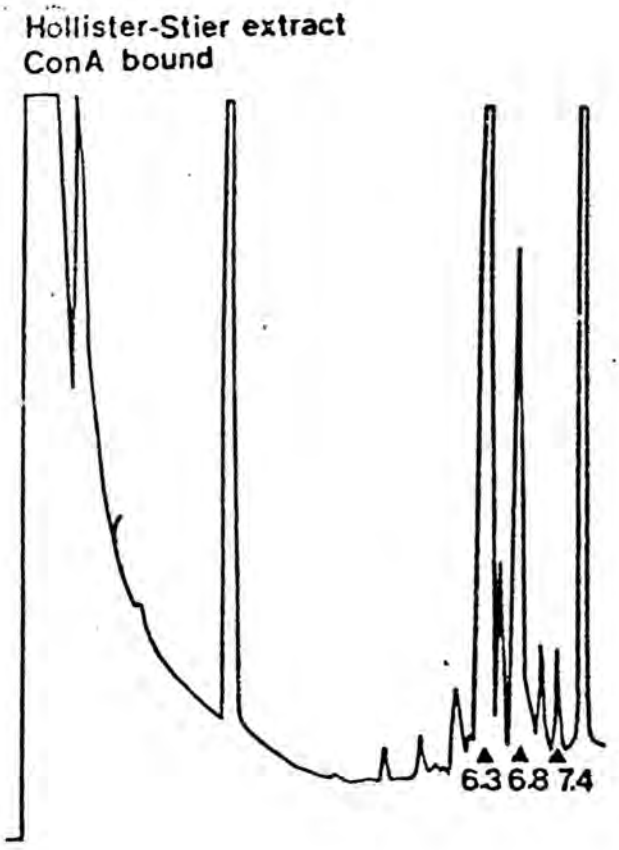
Figure 2

Gas chromatography profiles of the fractions eluted from ConA at neutral pH (ConA washout, Fraction I) and of the fractions bound to ConA, requiring elution with alpha-methyl mannoside (ConA bound, Fraction II) from the Hollister-Stier antigen. The separation of two control sugars (mannose and glucose) is also shown. The figures under each peak represent the distances measured in the graph between the beginning of the recording and the appearance of the peak. It can be seen that the major carbohydrate peaks in the ConA-bound material correspond to mannose.


mannose
6.3 6.8


glucose
7.0 7.4


Hollister-Stier extract
Con A washout
6.3 7.0 7.4


Hollister-Stier extract
Con A bound
6.3 6.8 7.4

Series I were done using CIE to compare reactions with the commercial Hollister-Stier extract and the purified mycelial antigens which we theorized to be the most specific for systemic infection based on the belief that the mycelial phase predominates as the organism's invasiveness progresses. Table 5 shows the results of these tests. Of 17 culture-positive patients included in Group I, 16 were found to be positive by CIE using commercial antigen, showing a greater sensitivity in this test than in double immunodiffusion, since eight patients of Group Ib were negative by the latter assay. However, this high frequency of positive results by CIE was obtained only with Fraction II (polysaccharide antigen) of the mycelial extract. With the cytoplasmic antigen, seven of nine Group Ia patients with clinical indications of systemic candidiasis, positive cultures, and positive results by double immunodiffusion test were positive, and no other patient or normal control showed reactivity. Of the two negative Group Ia patients, one was infected with a strain of C. tropicalis.

We also found that the ten patients in Group II with antibodies to fungi other than Candida whose sera showed precipitin lines when tested with Hollister-Stier antigen reacted exclusively with the polysaccharide fraction of the mycelial extract. The reactivity of the 14 positive normal control sera was similarly directed to polysaccharide antigens of C. albicans.

The fractions purified from the Hollister-Stier anti-

gen were tested on a more limited scale, but with similar results, in spite of the lesser degree of purity of these fractions. Of six sera tested, five were positive with the whole Hollister-Stier antigen and one negative (Table 6). Of the positive sera, one was a normal donor, one was a patient with antibodies to Histoplasma, and three were patients with clinical evidence of systemic candidiasis and cultures positive for Candida albicans. All five sera were positive when tested with Fraction II (polysaccharide antigen), whereas only the three from patients with suspected systemic candidiasis showed reactivity with Fraction I (cytoplasmic protein).

To complete our initial studies, selected patients' sera were tested by CIE for antibodies to yeast cytoplasmic and polysaccharide antigens to determine if any difference in reactivity was present depending on the growth phase of the organism (Table 7). With 19 sera tested, reactions with yeast polysaccharide were identical with reactions with mycelial polysaccharide. Three of the 19 who were negative with yeast cytoplasmic antigen were positive with mycelial cytoplasmic antigen.

Because of these promising initial studies, further investigation was done on another series of patients (Series II as defined in Materials and Methods). These patients' sera were tested by counterimmunoelectrophoresis against the unpurified Hollister-Stier antigen and against all four of the purified antigens prepared in our lab - the

TABLE 5

RESULTS OF CIE USING DIFFERENT ANTIGENS TO TEST SERA OF PATIENTS IN SERIES I

Patient group[a]	No.	Positive reactions by counterimmunoelectrophoresis		
		Hollister-Stier antigen	Mycelial antigen	
			Fraction I (cytoplasmic)	Fraction II (polysaccharide)
Ia	9	9	7	9
Ib	8	7	0	7
II	10	10	0	10
III	30	14	0	14

[a] See Materials and Methods for definition of patient groups in Series I.

TABLE 6

RESULTS OF CIE
USING FRACTIONATED HOLLISTER-STIER ANTIGEN

Patient	Crude	Fraction I	Fraction II
1	+	+	+
2	+	+	+
3	+	+	+
4	+	-	+
5	+	-	+
6	-	-	-

TABLE 7

RESULTS OF CIE STUDIES COMPARING RESPONSES TO YEAST AND MYCELIAL ANTIGENS

Patient	Antigens			
	Yeast		Mycelia	
	Cyto-plasmic	Poly-saccharide	Cyto-plasmic	Poly-saccharide
1	-	±	-	+
2	-	+	-	+
3	+	+	+	+
4	+	+	+	+
5	-	+	+	+
6	±	+	±	+
7	-	+	-	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	-	-	-	-
12	-	+	-	+
13	-	+	+	+
14	+	+	+	+
15	+	+	+	+
16	-	+	+	+
17	-	+	-	+
18	-	-	-	-
19	-	-	-	-

yeast cytoplasmic and polysaccharide antigens and the mycelial cytoplasmic and polysaccharide antigens. Table 8 shows the results of CIE tests on these patients' sera.

All of the sera from the groups with proven or probable disseminated candidiasis reacted with the unpurified Hollister-Stier antigen but, in addition, reactions were obtained with two of three patients with localized infection, three of nine with vaginal candidiasis, four of nine with mucocutaneous candidiasis, and 14 of 30 normals as had been shown in Series I. Reaction with the yeast and mycelial polysaccharide antigens occurred in 11 of 12 patients in Groups I and II but also was seen in 14 of the normals and in five of the ten in Groups III to VI who had reacted with the unpurified antigen. Reaction with the cytoplasmic antigens was much more specific. Seven out of seven patients with proven systemic candidiasis reacted with the yeast cytoplasmic antigen and four out of seven with the mycelial cytoplasmic antigen. The three who did not react with mycelial cytoplasmic antigen all had C. tropicalis infections rather than C. albicans. Only one patient in any of the other groups reacted with either cytoplasmic antigen. That patient had a long-standing chronic mucocutaneous candidiasis. An interesting additional note is that three of the patients with disseminated disease who had serial samples collected had only anti-polysaccharide antibodies when first studied but developed anti-cytoplasmic antibodies as their disease progressed (Table 9).

TABLE 8

RESULTS OF CIE USING DIFFERENT ANTIGENS

Patient Group[a]	No.	Positive reactions with[b] H-S	yc	yp	mc	mp
I	7	7	7	7	4	6
II	5	5	2	4	2	5
III	3	1	0	1	0	0
IV	3	2	0	2	0	1
V	9	3	0	0	0	0
VI	9	4	0	2	1	3

[a] See Materials and Methods for definition of patient groups.

[b] H-S = Hollister-Stier antigen; yc = yeast cytoplasmic antigen; yp = yeast polysaccharide antigen; mc = mycelial cytoplasmic antigen; mp = mycelial polysaccharide antigen.

TABLE 9

APPEARANCE OF ANTI-CYTOPLASMIC ANTIBODY IN PATIENTS WITH SYSTEMIC CANDIDIASIS

Date	Patient LM		Date	Patient LS		Date	Patient ET	
	Cyto	Poly		Cyto	Poly		Cyto	Poly
11/4	-	+	5/29	-	+	6/5	-	+
12/19	-	+	6/6	-	+	6/11	+	+
1/6	-	+	6/20	-	+			
1/21	+	+	7/7	-	+			
2/23	+	+	7/31	+	+			

Comparison of Reactions with the Various Antigens Using QIF

Sera from 30 patients were tested by quantitative immunofluorescence for antibodies to all of the purified antigens, and sera from 10 healthy controls were tested in the same way to obtain a range of normal values (Table 10). Patients 1-14 fit our criteria (see Materials and Methods) for a diagnosis of systemic candidiasis. Patients 15 - 28 had local or mucocutaneous infection and 29 and 30 were infected with other fungi. The Mann-Whitney test was used to analyze differences between the mean values for the systemic, local and mucocutaneous, and normal groups (Table 11).

It can be seen (Table 10) that the mean normal antibody level to the polysaccharide antigens is higher than that to the cytoplasmic antigens and the standard deviation is substantially larger. This reflects the presence of antibodies to cell wall mannan of yeasts in many normal individuals as was seen in our CIE data. However, statistical analysis (Table 11) of the difference between quantitated levels of antibody in the normal group and in a group of systemic patients showed that use of polysaccharide antigens in a quantitative technique can be quite useful in diagnosing systemic disease. The level of significance of the difference between the two groups with the yeast polysaccharide antigen was $p = 0.0003$ and with the mycelial polysaccharide was $p < 0.0001$.

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Looking at individual patients to see if their quantitated antibody levels were greater than two standard deviations above the normal mean (Table 12) shows that the mycelial polysaccharide antigen was quite sensitive for detecting systemic disease with 13 out of 14 patients having values above the cut-off. The one remaining patient had a value of 25.5, very near the cut-off of 26.8. The yeast polysaccharide antigen was a little less sensitive, however, picking up nine out of 14 patients with systemic disease.

Analysis of the differences between the systemic group and the local and mucocutaneous group again showed the polysaccharide antigen to be quite useful in the quantitative test. The level of significance of the difference with yeast polysaccharide was $p = 0.0004$ and with mycelial polysaccharide was $p < 0.0001$. Analysis of the difference in antibody levels between the local and mucocutaneous group and the normals showed no significant difference with either of the polysaccharide antigens or with the mycelial cytoplasmic antigen, assuming a $p < 0.05$ level to be necessary for significance.

Statistical analysis of the differences between the levels of anti-cytoplasmic antibody in the three groups revealed a generally less significant difference than was found with the anti-polysaccharide antibodies, with the exception that yeast cytoplasmic antibodies revealed the only significant difference between the local

and mucocutaneous group and the normals. The level of that difference was $p = 0.0401$. The difference between the systemic and normal groups with yeast cytoplasmic antigen was significant at a level of $p = 0.0089$ as compared with the yeast polysaccharide antigen at a level of $p = 0.0003$. The difference with the mycelial cytoplasmic antigen was significant at a level of $p = 0.0268$ as compared with the $p < 0.0001$ level found with mycelial polysaccharide. The differences between the local and mucocutaneous group and the systemic group were not significant at a $p < 0.05$ level with yeast cytoplasmic while with mycelial cytoplasmic there was significance at a level of $p = 0.0409$. It is interesting that patients 12, 13, and 14 were classified as systemics on the basis of multiple positive blood cultures but had no proven deep focus of infection, and these three patients had low values of anti-cytoplasmic antibodies as compared with the majority of the patients (#1-11) who had a proven deep focus of infection. Patient #11 who also had low anti-cytoplasmic antibodies had a very rapid progression to death from the beginning of his disease. Looking at Table 12, these four patients and one other were the only five systemic patients who did not have levels greater than two standard deviations above the normal mean with mycelial cytoplasmic antigen.

Patients 29 and 30 who had other fungal infections and who had anti-polysaccharide antibodies by CIE did not have antibody levels greater than two standard de-

viations above the normal mean against any of the antigens when tested quantitatively.

III. Use of QIF to Determine Antibody Class

The quantitative immunofluorescence assay which had been developed by us and used to measure concentrations of antibody to various types of Candida antigen could be modified for other uses as well. One which was investigated was the determination of class of antibody, which would be easily accomplished by changing the specificity of the fluorescent antisera so that antibodies of only one class would be determined. This modification was used to test the hypothesis that superficial forms of Candida infection might lead to the production of antibodies of the IgA class in higher titer than, or to the exclusion of, other classes of antibody due to the association of IgA with mucosal epithelium. It was hoped that, if this were true, it might aid in differentiation between superficial and deep-seated infections. Earlier studies on this question (32, 90) had been done using an indirect immunofluorescence test on smears of Candida albicans cells and had lead to conflicting results.

We used our group of vaginal candidiasis patients (see Materials and Methods for diagnostic criteria) and tested the sera first for precipitating antibodies to the Hollister-Stier extract of C. albicans and to purified cell wall polysaccharide since that antigen is the primary one

TABLE 10

RESULTS OF QIF STUDIES OF RESPONSES TO CYTOPLASMIC AND POLYSACCHARIDE ANTIGENS OF YEAST AND MYCELIA [b]

Patient [a]	Antigens			
	Yeast		Mycelia	
	Cytoplasmic	Polysaccharide	Cytoplasmic	Polysaccharide
1	7.6	28	17.8	66.2
2	9.5	45.2	50.0	102.5
3	3.2	22.3	7.6	33.8
4	14.6	16.6	62.4	66.2
5	8.1	49.7	26.8	89.8
6	6.4	28.7	14.6	59.2
7	6.4	17.2	20.4	25.5
8	8.3	12.1	29.9	32.5
9	5.8	15.9	22.9	26.8
10	26.8	96.8	29.0	96.0
11	2.5	25.2	2.5	49.1
12	14.8	16.6	5.6	50.7
13	4.8	117.2	4.5	>127
14	6.4	27.7	4.1	57.0
15	4.8	13.1	3.5	34.1
16	3.8	15.6	4.1	30.9
17	5.1	10.0	6.4	10.7
18	7.6	59.7	9.6	54.7
19	8.3	13.4	15.3	16.6
20	2.2	6.1	4.8	15.6
21	7.0	5.3	7.6	4.7
22	8.3	8.0	10.2	5.3
23	5.1	7.3	7.6	7.3
24	4.4	6.6	8.3	6.0
25	5.7	16.7	8.9	16.7
26	5.1	17.3	7.6	14.7
27	4.4	14.7	6.4	6.0
28	10.8	6.0	19.1	4.0
29	1.9	4.4	2.5	6.4
30	3.8	14.0	6.4	21.0
Mean of 10 normal	4.7	9.4	6.6	13.2
Standard deviation	1.6	5.6	2.7	6.8

[a] See text for identification of patients.

[b] Underlined values are greater than 2 standard deviations above the normal mean.

TABLE 11

LEVEL OF SIGNIFICANCE OF DIFFERENCES
BETWEEN GROUPS BY THE MANN-WHITNEY TEST

	Normals				Local and Mucocutaneous Patients			
	Yeast		Mycelial		Yeast		Mycelial	
Antigens	Cyto	Poly	Cyto	Poly	Cyto	Poly	Cyto	Poly
Systemic Patients	0.0089	0.0003	0.0268	<0.0001	0.0655	0.0004	0.0409	<0.0001
Local and Mucocutaneous Patients	0.0401	0.1210	0.1075	0.3859	-	-	-	-

TABLE 12

NUMBERS OF PATIENTS WITH QIF VALUES GREATER THAN 2 STANDARD DEVIATIONS ABOVE THE NORMAL MEAN WITH EACH ANTIGEN

Group	No. in Group	Antigen			
		Yeast		Mycelial	
		Cytoplasmic	Polysaccharide	Cytoplasmic	Polysaccharide
Systemic Patients	14	6	9	9	13
Local and Mucocutaneous Patients	14	3	1	2	3
Normals	10	1	0	1	0

detected in the indirect immunofluorescence test which had previously been used in these studies. Three out of 17 patients in the vaginal candidiasis group showed precipitating antibodies to the commercially prepared extract but none demonstrated precipitating antibodies to purified mannan.

Quantitative immunofluorescence, using polyvalent antisera to human immunoglobulins, was used to test the sera of 16 patients with Candida vaginitis for antibodies to the purified yeast polysaccharide antigen. Table 13 shows the detected levels of antibody, expressed as a percentage of the maximum reading found with a highly positive control sera, and the mean level found in ten normal controls. Statistical comparison of the antibody levels in the patient and normal groups, using the Mann-Whitney test, showed that the means of the two groups differed at a level of $p = 0.0778$. If $p < 0.05$ is chosen as the level necessary for statistical significance, the antibody levels in these two groups do not show a significant difference.

Eight of the patients had antibody levels greater than one standard deviation above the mean level of the normal controls (that is above 15%). Quantitation of antibody of the IgG class and the IgA class was done in these patients using monospecific antisera to human IgG and IgA in the fluoroimmunoassay. Results (Table 13) showed the antibody to be predominantly IgG in all cases.

Indirect immunofluorescence was done on these

same eight sera, with the serum from a systemic candidiasis patient used as a positive control and serum from a normal individual used as a negative control. The intensity of fluorescence with fluorescein-labeled anti-human IgG following incubation with a vaginal candidiasis patient's serum was indistinguishable from the intensity with the systemic candidiasis patient's serum (Figure 3). Intensity with fluorescein-labeled anti-human IgA was definitely less than that with anti-IgG, and less than that of the systemic candidiasis patient with anti-IgA. The normal showed only a trace of fluorescent staining with anti-IgG and was negative with anti-IgA.

These studies on vaginal candidiasis patients served not only to support the earlier data (90) that had indicated that anti-Candida antibodies in vaginal candidiasis patients were predominantly IgG but also to demonstrate the usefulness of the QIF assay to achieve a number of different goals in antibody detection. Not only can the presence and concentration of antibody be determined, but their class is easily defined as well.

IV. Antigen Detection Studies

Having completed these studies of detection of antibodies to Candida albicans, our attention was turned to attempts to detect circulating antigen.

In order to have sufficient amounts of sera, which should contain circulating antigen, for the investi-

TABLE 13

RESULTS OF STUDIES USING QUANTITATIVE IMMUNOFLUORESCENCE AND INDIRECT IMMUNOFLUORESCENCE TO DETERMINE LEVELS AND CLASS OF ANTI-*CANDIDA* ANTIBODIES IN VAGINAL CANDIDIASIS PATIENTS

PATIENT	QIF			IIF	
	TOTAL ANTIBODY	IgG ANTIBODY	IgA ANTIBODY	IgG	IgA
P.H.	6.1%	N.D.[1]	N.D.	N.D.	N.D.
S.S.	36.3%	15.2%	8.6%	3+	2+
R.P.	0.3%	N.D.	N.D.	N.D.	N.D.
D.B.	8.0%	N.D.	N.D.	N.D.	N.D.
S.G.	7.3%	N.D.	N.D.	N.D.	N.D.
M.D.	6.6%	N.D.	N.D.	N.D.	N.D.
H.M.	16.7%*	7.5%	4.5%	3+	1+
C.W.	17.3%*	6.2%	1.2%	3+	1+
C.H.	14.7%	N.D.	N.D.	N.D.	N.D.
G.B.	4.9%	N.D.	N.D.	N.D.	N.D.
P.D.	27.7%	14.6%	4.3%	3+	1+
M.E.	18.7%*	10.3%	5.2%	3+	1+
A.C.	20.3%	10.7%	3.4%	3+	1+
C.M.	17.7%	8.5%	2.7%	2+	1+
M.B.	23.3%	11.1%	5.2%	3+	1+
W.B.	4.7%	N.D.	N.D.	N.D.	N.D.
Mean	14.7%	10.5%	4.4%		
Standard Deviation	9/4%	3.2%	2.2%		
Mean of 10 Normals[2]	9.4%				
Standard Deviation	5.5%				

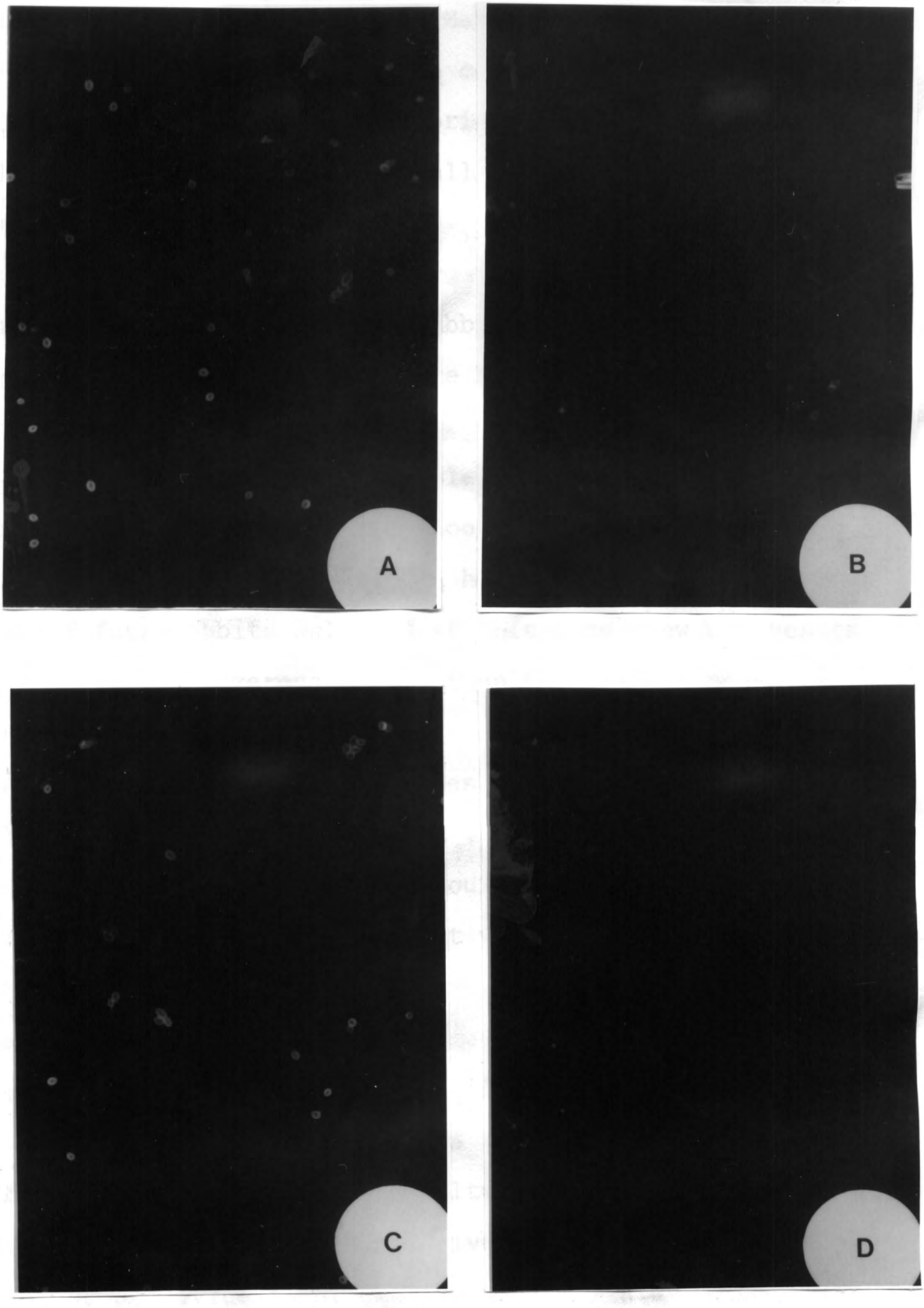
[1] N.D. = Not Done

[2] Three were positive by CIE; the sera from an asymptomatic donor found to have high antibody titers was not included in this series.

(*) Positive by CIE.

Figure 3

Indirect immunofluorescence on C. albicans cells: (A) sera of a systemic candidiasis patient followed by fluorescent anti-human IgG; (B) sera of a systemic candidiasis patient followed by fluorescent anti-human IgA; (C) sera of a vaginal candidiasis patient followed by fluorescent anti-human IgG; (D) sera of a vaginal candidiasis patient followed by fluorescent anti-human IgA.


A
B
C
D

gation of these assays, Candida endocarditis was induced in 13 rabbits as described in Materials and Methods. Nine were infected with 10^6 Candida cells, two with 10^7 and two with 10^8. Rabbits were bled prior to infection, four at 2½ hours post-infection, and all on days one and two post-infection and every two to three days thereafter until death or sacrifice. To determine the extent of infection achieved in these rabbits, blood cultures were done each time the rabbits were bled and tissue was cultured and examined grossly and microscopically at death or sacrifice of the animals. Table 14 summarizes the culture and pathology results. All blood cultures were negative except the samples drawn at 2½ hours post-infection. Four out of four rabbits cultured at this time grew out yeasts that were too numerous to count on the culture plate. Gross examination of the hearts of the animals revealed vegetations on the aortic valves and/ or the ventricles of all rabbits except one which died two days post-infection. Another which died within 24 hours showed damage to the valves but no truly significant vegetations. Figure 4 shows a vegetation from the left ventricle of one of the infected rabbits. Gross examination of the kidneys revealed microabscesses and/or infarcts in all animals except the ones which died within 24 hours and at two days. Eight of the 13 rabbits had positive cultures from the cardiac vegetations and nine had positive cultures from the kidneys. Microscopic evidence of Candida growth was seen on sections

taken from the hearts of six rabbits. Figure 5 is an example of what was seen. Microscopic evidence of Candida growth was seen on sections taken from the kidneys of three animals. Figure 6 is an example of a rabbit kidney in which organisms are seen in tubules.

From the evidence shown above, it seems obvious that at least 10 of the 13 rabbits in whom induction of Candida endocarditis was attempted developed disseminated disease. Therefore, their sera should have provided a good source of material in which to detect circulating antigen. We used counterimmunoelectrophoresis as described in Materials and Methods to test each sample from these rabbits and were unable to detect antigen in any of them. In addition, the QIF inhibition assay for antigen was used to test some samples. A standard curve as seen in Figure 7 was obtained with a known antigen solution, but no samples of rabbit sera showed any inhibition at all.

A limited number of patient samples were tested for antigen by CIE, QIF inhibition, and ELISA inhibition. Eight were tested by CIE including five classified as having systemic candidiasis and three with local or mucocutaneous candidiasis. No antigen was discovered in the sera of any of these patients, although CIE showed one patient to have antigen present in his joint fluid.

The QIF inhibition assay was used to test the sera of 12 normals and eight patients including four classified as having systemic candidiasis and four with

TABLE 14

CULTURE AND PATHOLOGY RESULTS ON RABBITS INFECTED WITH CANDIDA ALBICANS

Rabbit	No. of Infecting Organisms	Days Lived Post Infection	Gross Organ Involvement		Culture Results		Microscopic Presence of Organisms	
			Heart	Kidney	Heart	Kidney	Heart	Kidney
1	10^8	1	+	-	+	+	-	-
2	10^8	7	+	+	+	+	+	-
3	10^7	21	+	+	+	-	+	-
4	10^7	21	+	+	+	+	+	-
5	10^6	2	-	-	-	-	-	-
6	10^6	29	+	+	-	+	-	+
7	10^6	18	+	+	+	+	+	+
8	10^6	24	+	+	-	-	-	-
9	10^6	29	+	+	+	+	+	-
10	10^6	31	+	+	+	+	+	+
11	10^6	32	+	+	+	+	-	-
12	10^6	38	+	+	-	-	-	-
13	10^6	29	+	+	-	+	-	-

Figure 4

Left ventricular vegetation seen in a rabbit injected with 10^6 Candida albicans cells.

Figure 5

Candida organisms seen in a microscopic section of a cardiac vegetation from a rabbit infected with 10^6 Candida cells.

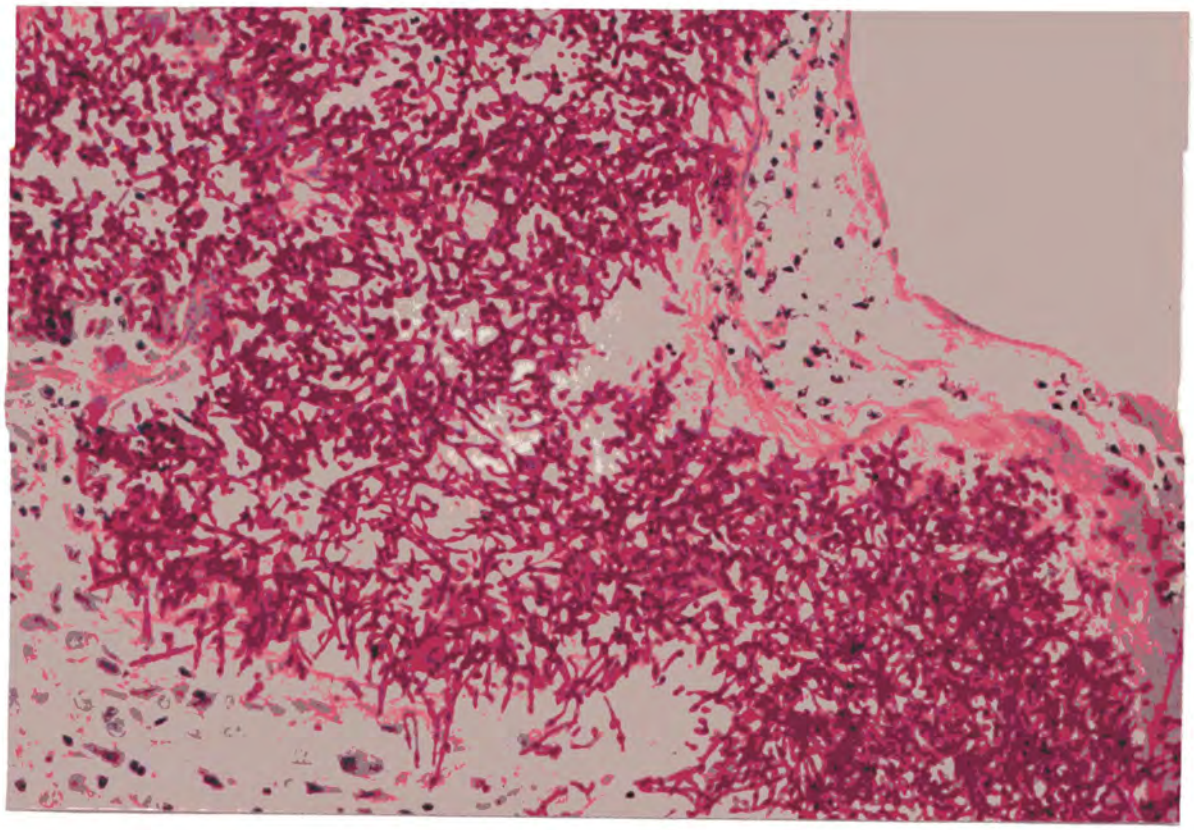
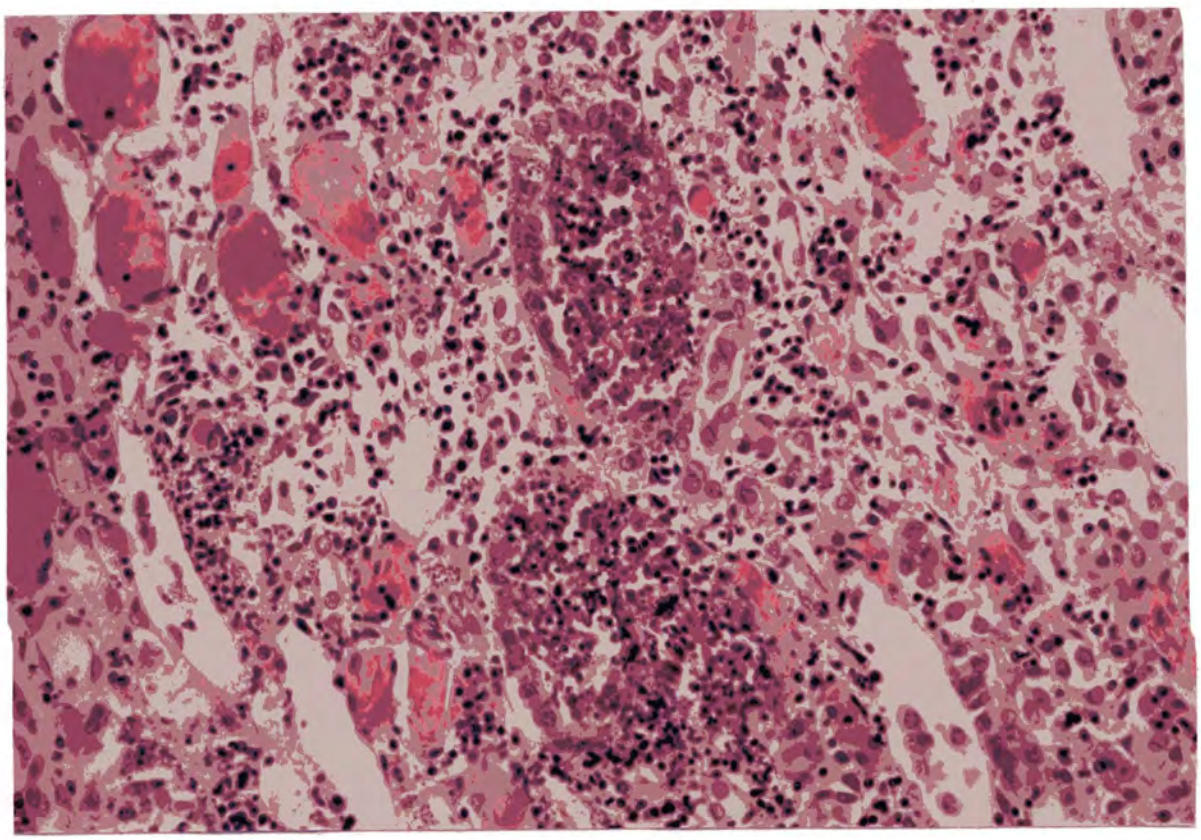


Figure 6

Kidney section from a rabbit infected with 10^6 Candida albicans cell.



local or mucocutaneous infection. These sera were all treated as described in Materials and Methods to break up any antigen-antibody complexes and destroy any free antibody. For each sera a control was run to be certain that no free antibody remained to interfere with the inhibition assay. The percentage inhibition of each sera can be seen in Table 15 and comparison of the normals and patients reveals no significant difference. Antigen was, therefore, undetectable in these samples by this method.

The ELISA inhibition assay was used to test ten normals and these same eight patients. The sera were treated as above. An inhibition curve with standard amounts of antigen can be seen in Figure 8. The amount of inhibition seen with normals and patients is seen in Table 16. Again the difference between patients and normals was insignificant and no antigen could be detected by this method.

We also tried to determine the effect of the sodium hydroxide treatment on C. albicans polysaccharide antigen. For this serial dilutions of purified antigen were added to a fixed volume of normal human serum and the mixture was submitted to the treatment as suggested by Segal, et al. (57) and described in Materials and Methods. When the treated mixture was tested in the QIF inhibition assay, the standard inhibition curve normally seen with such serial dilutions of antigen was not obtained.

TABLE 15

RESULTS OF THE QIF-INHIBITION ASSAY FOR ANTIGEN DETECTION

Normals	% Inhibition	Patients [a]	% Inhibition
1	10.7	1	0
2	5.8	2	0
3	0	3	0
4	2.9	4	2.1
5	0	5	5.2
6	0	6	10.3
7	1.0	7	17.5
8	0	8	0
9	12.6		
10	2.9		
11	1.0		
12	0		

[a] Patients 1-4 were diagnosed as having systemic candidiasis.

Figure 8

Standard curve for the ELISA inhibition assay for *Candida* antigen.

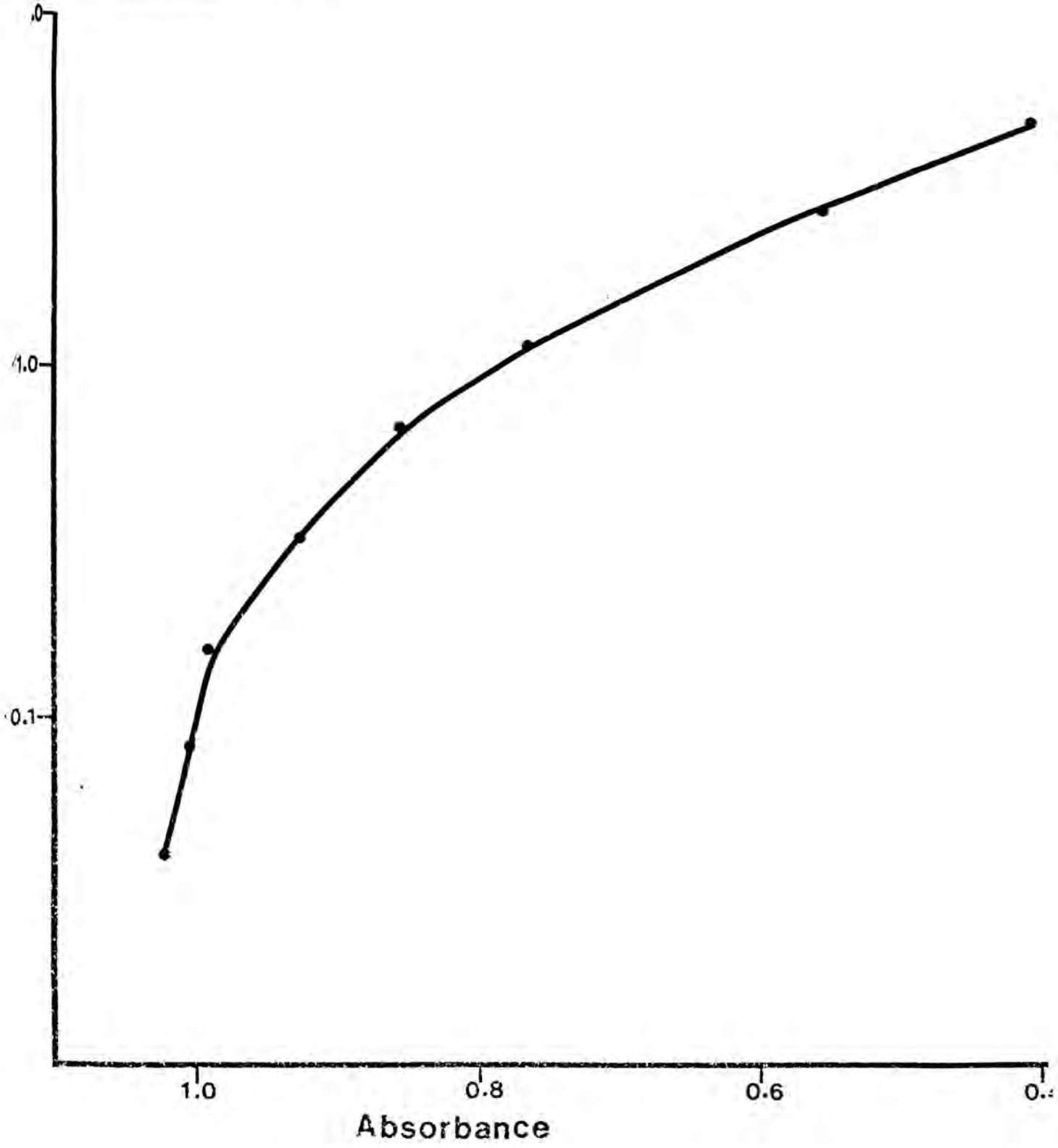


TABLE 16

RESULTS OF THE ELISA INHIBITION ASSAY FOR ANTIGEN DETECTION

Normals	% Inhibition	Patients [a]	% Inhibition
1	5.3	1	0
2	0	2	2.2
3	0.5	3	9.3
4	0	4	14.3
5	11.8	5	3.4
6	11.2	6	14.8
7	11.8	7	5.3
8	12.3	8	14.9
9	11.0		
10	15.3		

[a] Patients 1-4 were diagnosed as having systemic candidiasis.

V. Immune Complex Studies

Because of the lack of success with these antigen detection techniques, our attention was focused on investigation of the possibility that circulating immune complexes might be present in the sera of systemic candidiasis patients and that they might be interfering with antigen detection. Sera from eleven patients in our group with disseminated candidiasis and from 18 normal controls was tested by four immune complex screening tests - the Direct Laser Nephelometry test, the PEG-C_4, the PEG-IgG and the C_1q tests. The scores on the individual tests were compared for the two groups and, after the individual scores were compiled into IC scores, those, too, were compared for the two groups. A statistically significant difference ($p < 0.05$) was found between the patient and normal populations for three of the four screening methods (all but the C_1q assay) and with the IC scores (Table 17).

With the results of these screening tests substantiating the existence of circulating immune complexes in the sera of patients with systemic candidiasis, attempts were begun to isolate and characterize the complexes from several patients.

Sera from four patients from our group with systemic candidiasis, from one patient with a severe and chronic mucocutaneous candidiasis, from one normal individual, and from one patient with vasculitis who had high immune complexes and no sign of any Candida infection were analyzed.

Immune complexes were isolated as described in Materials and Methods. A characteristic profile from an ACA 34 separation of the PEG precipitate of the sera of a patient with systemic candidiasis is shown in Figure 9. Peaks 1, the high molecular weight peak, and 2, the low molecular weight peak, were pooled and analyzed for the presence of Candida antigen, anti-Candida antibodies, IgG, IgM, IgA, C_1q, C_3, and C_4. The high molecular weight peak was applied to a Sepharose 4B-Protein A column and eluted first with PBS to obtain the bound protein fraction. The bound fraction was assumed to contain IgG which had stuck to the Protein A. An elution profile is seen in Figure 10. These fractions were analyzed as had been the ACA peaks.

Table 18 shows the results of the analysis of the isolated fractions of patient and control sera. Samples 1-4 were obtained from patients classified by our criteria as having systemic candidiasis. Sample 5 was from a patient with severe and chronic mucocutaneous candidiasis. Sample 6 was obtained from a normal individual with no sign of any Candida infection. Sample 7 was from a patient with vasculitis who had elevated circulating immune complexes but no evidence of Candida infection. Analysis of the sera of all the investigated individuals showed no evidence of antigen by counterimmunoelectrophoresis but antibody was present in all sera except the normal individual by both CIE and quantitative immunofluorescence.

TABLE 17

LEVELS OF CIRCULATING IMMUNE COMPLEXES IN SYSTEMIC CANDIDIASIS PATIENTS BY FOUR SCREENING TECHNIQUES (DIRECT NEPHELOMETRY, C_1q ASSAY, C_4 ASSAY AND IgG ASSAY) AND A COMBINED SCORE

Patient	DL	C_1q	C_4	IgG	IC Score
1	17	79.1	42	0.45	3.1700
2	18	99.5	41	0.70	2.6367
3	4	109.2	28	0.30	0.7756
4	8	75.6	-	0.64	1.6747
5	5	-	42	0.45	2.2791
6	8	138.0	35	0.22	1.8800
7	12.5	110.0	23	0.42	1.9611
8	6	89.9	34	0.78	3.1686
9	0	101.6	39	0.39	0.9762
10	0	105.0	20	0.77	1.3651
11	5	126.8	0	0.13	0.0084
Mean of the Patients	7.59	103.47	30.4	0.48	1.8086
Mean of 18 Normals	2.3	100.0	19.23	0.21	0.0433
p Value of Significance of Difference Between Patients and Normals	0.0057	0.1635	0.0068	0.0001	<0.0001

When the ACA 34 peaks were analyzed, anti-Candida antibody was found in the low molecular weight fraction of all those examined except the normal and sample 7 and in the high molecular weight fraction of all except the normal and sample 1. Sample 1 was the first sera tested and was less extensively analyzed, having been tested for antibody only by CIE, which is less sensitive than QIF, and having not been analyzed for immunoglobulin or complement components. Analysis of the ACA peaks by CIE for antigen, however, revealed its presence in the high molecular weight fractions of samples 2 and 5 and in the low molecular weight fractions of samples 1 and 2. Figure 11 is a photograph of the CIE plate testing sample 2's fractions for antigen.

Analysis of the ACA 34 fractions for immunoglobulin and complement components was done by immunodiffusion. The analysis of sample 4 is shown in Figure 12 as an example. The three systemic candidiasis patients tested, the mucocutaneous patient, and the patient with vasculitis all had three immunoglobulin classes present in the high molecular weight fraction while the normal individual had evidence only of IgM. Complement components were found in the high molecular weight fraction only in the patients with systemic candidiasis.

Analysis of the bound and unbound fractions from the Protein A column separation of the high molecular weight peaks of each sera revealed antibody in the bound fraction only of samples 2, 3, and 5, two of which were

Figure 9

Elution profile from ACA 34 column of 5% PEG precipitate of serum from a patient with systemic candidiasis.

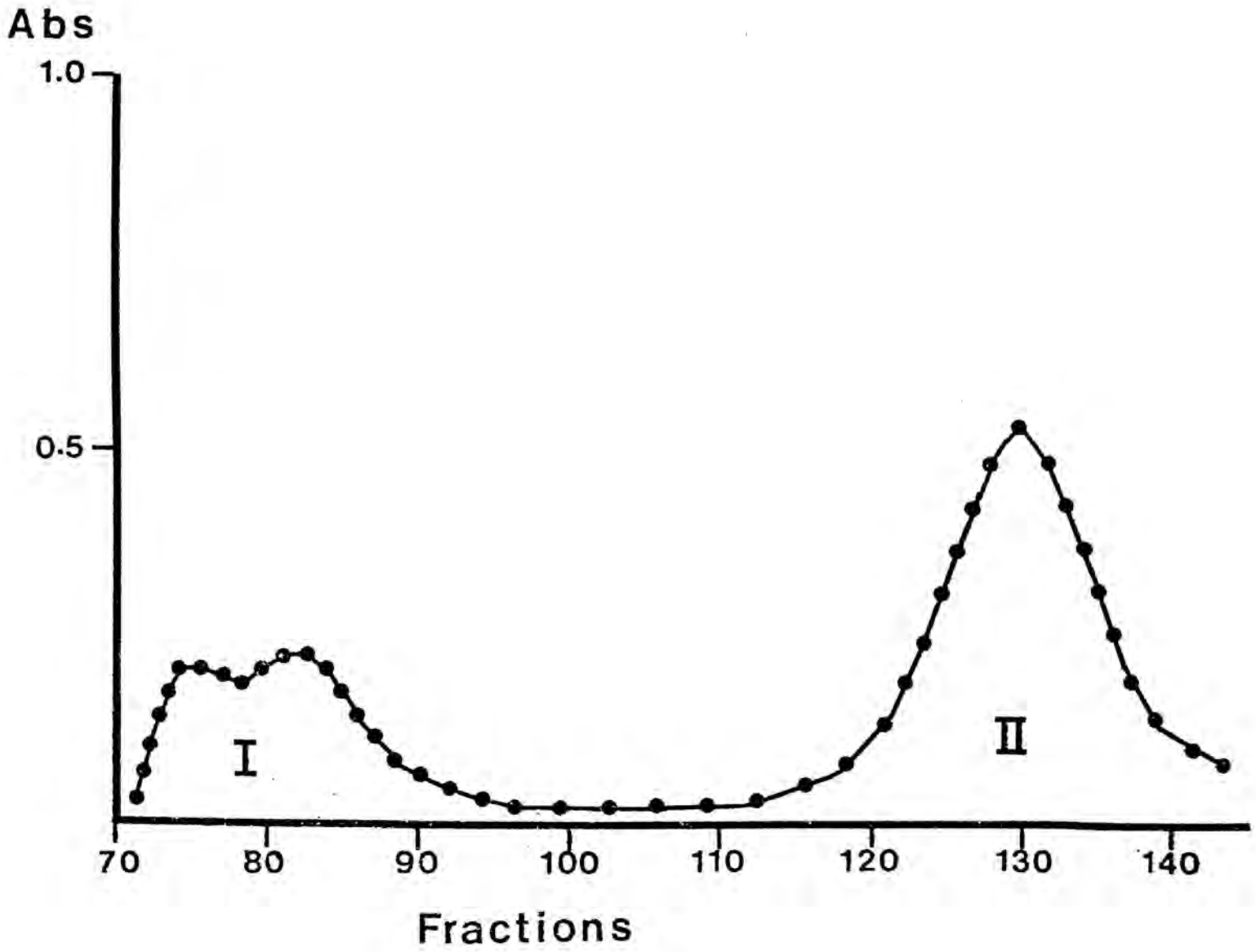


Figure 10

Elution profile from a Sepharose 4B Protein A column to which the high molecular weight fraction of sera from a patient with systemic candidiasis was applied.

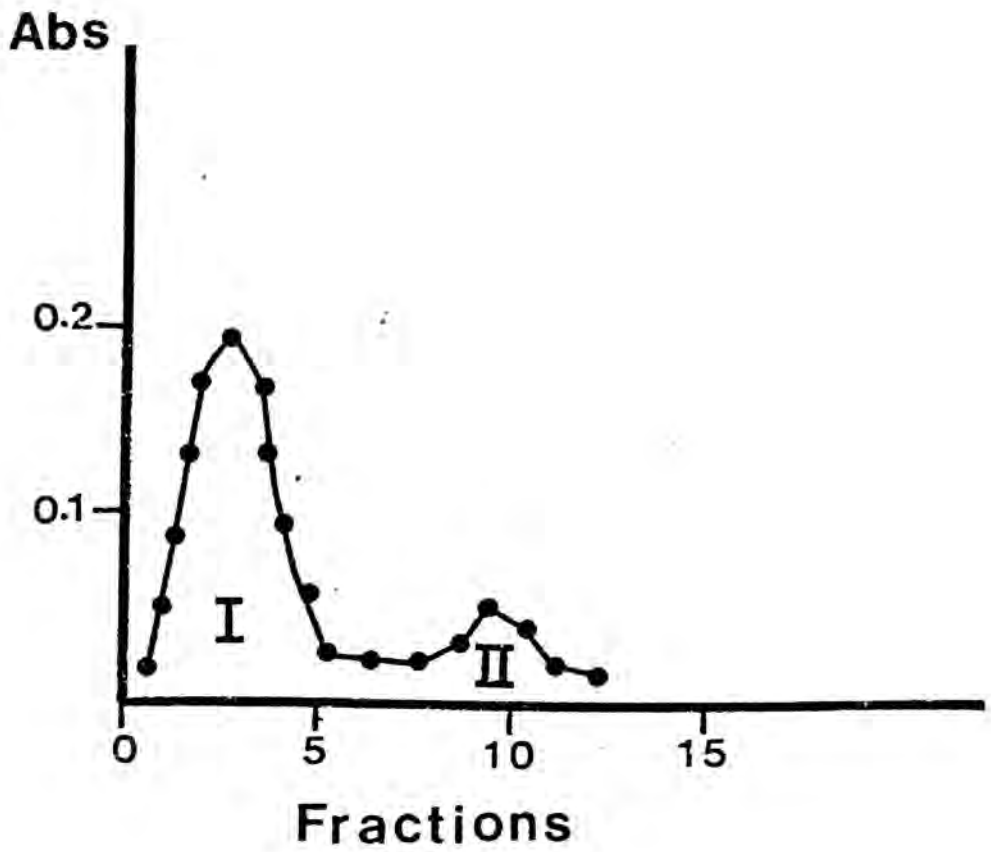


TABLE 18

RESULTS OF THE ANALYSIS OF FRACTIONS OF PATIENT AND CONTROL SERA ISOLATED FOR IMMUNE COMPLEXES

Fractions	Sera	ACA-34 high mol. wt. frac.									ACA-34 low mol. wt. frac.								Protein A bound frac.								Protein A unbound frac.							
Sample[a]	Ag	Ab	Ag	Ab	G	M	A	C1q	C3	C4	Ag	Ab	G	M	A	C1q	C3	C4	Ag	Ab	G	M	A	C1q	C3	C4	Ag	Ab	G	M	A	C1q	C3	C4
1	-	+	-	-							+	+							-	-							+	-						
2	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	+	-	+	+	+	-	-	+
3	-	+	-	+	+	+	+	+	-	-	-	+	+	-	+	-	+	+	-	+	-	-	-	+	-	+	-	+	-	-	-	-	-	-
4	-	+	-	+	+	+	+	-	-	+	-	+	+	-	+	-	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	-	+	-
5	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	-						
6	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-

[a] Samples 1-4 were from patients diagnosed as having systemic candidiasis.

Sample 5 was from a patient with severe and chronic mucocutaneous candidiasis.

Sample 6 was from a normal individual.

Sample 7 was from a patient with elevated immune complexes and no sign of Candida infection.

from systemics and one from the mucocutaneous patient. Antibody was found in the unbound fraction of samples 3 and 4 from systemics and in the sample from the patient with vasculitis. The latter fact, along with the evidence of IgM and IgA in sample 7's unbound fraction but not IgG, made it seem that the antibody to Candida which had appeared in his high molecular weight peak might have been IgM rather than an immune complex. Antigen was found in the bound fraction from samples 2 and 5 and in the unbound fraction of samples 1 and 2. Immunoglobulins G, M, and A were detected in the bound fraction of sample 5 and G and M were found in the bound fraction of sample 4. Immunodiffusion may not have been sensitive enough to detect the amount of immunoglobulin in sample 2 although more sensitive methods could detect specific antibody. C_1q was detected in the bound fraction of samples 3 and 5 and C_4 also in sample 3, further substantiating the existence of immune complexes in these patients. The unbound fraction from samples 2 and 4 contained G, M, and A and C_4 in sample 2 and C_3 in sample 4.

The Protein A-bound fraction in sample 5 which had been found to contain both antigen and antibody was applied to another ACA 34 column, this time equilibrated and eluted in an acid buffer to try to keep the antigen and antibody dissociated. The profile can be seen in Figure 13. Antibody was found in Peak II by QIF and antigen was found in Peaks III and V by CIE. A reproduction of this CIE

Figure 11

Counterimmunoelectrophoresis testing ACA 34 fractions from a patient with systemic candidiasis for Candida antigen.

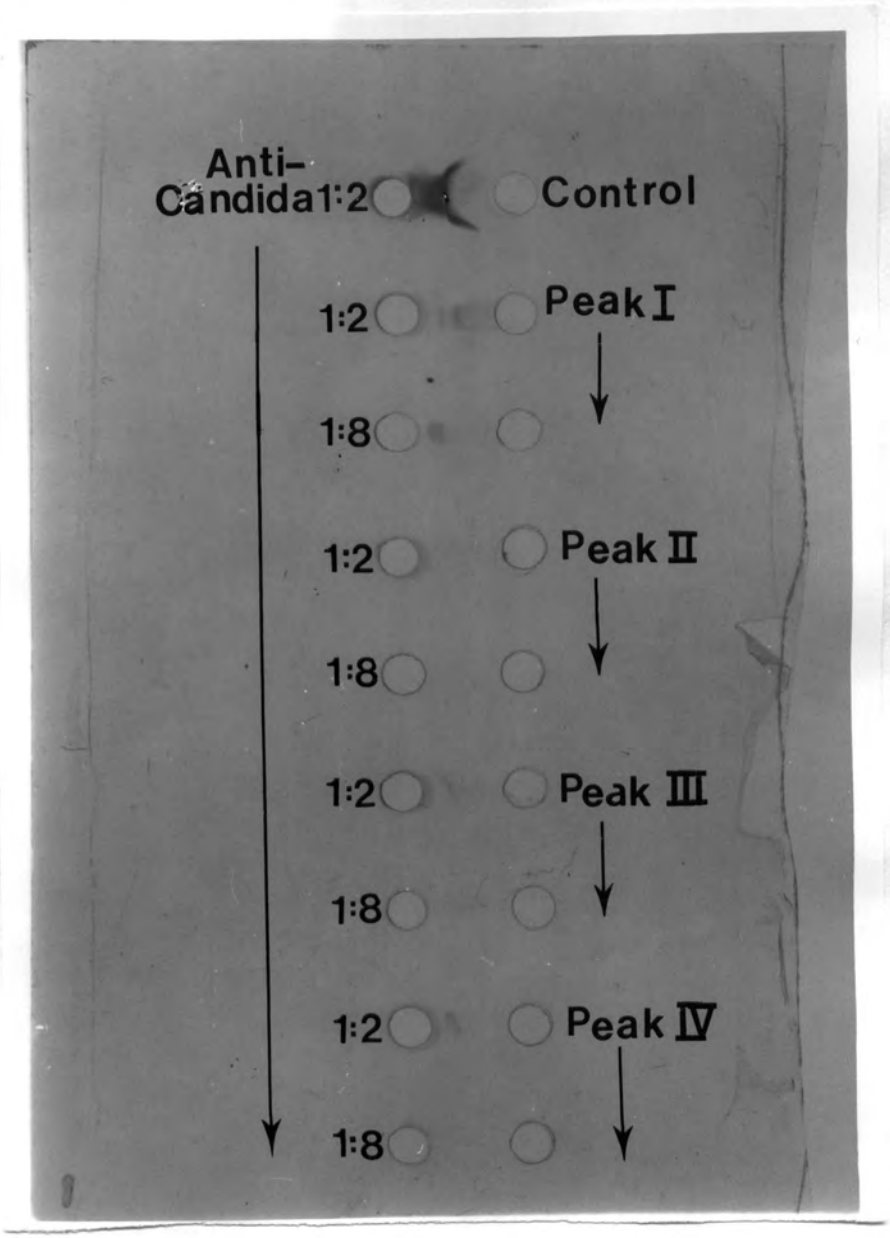


Figure 12

Immunodiffusion to determine the immunoglobulin and complement component content of the ACA 34 fractions from the sera of a patient with systemic candidiasis.

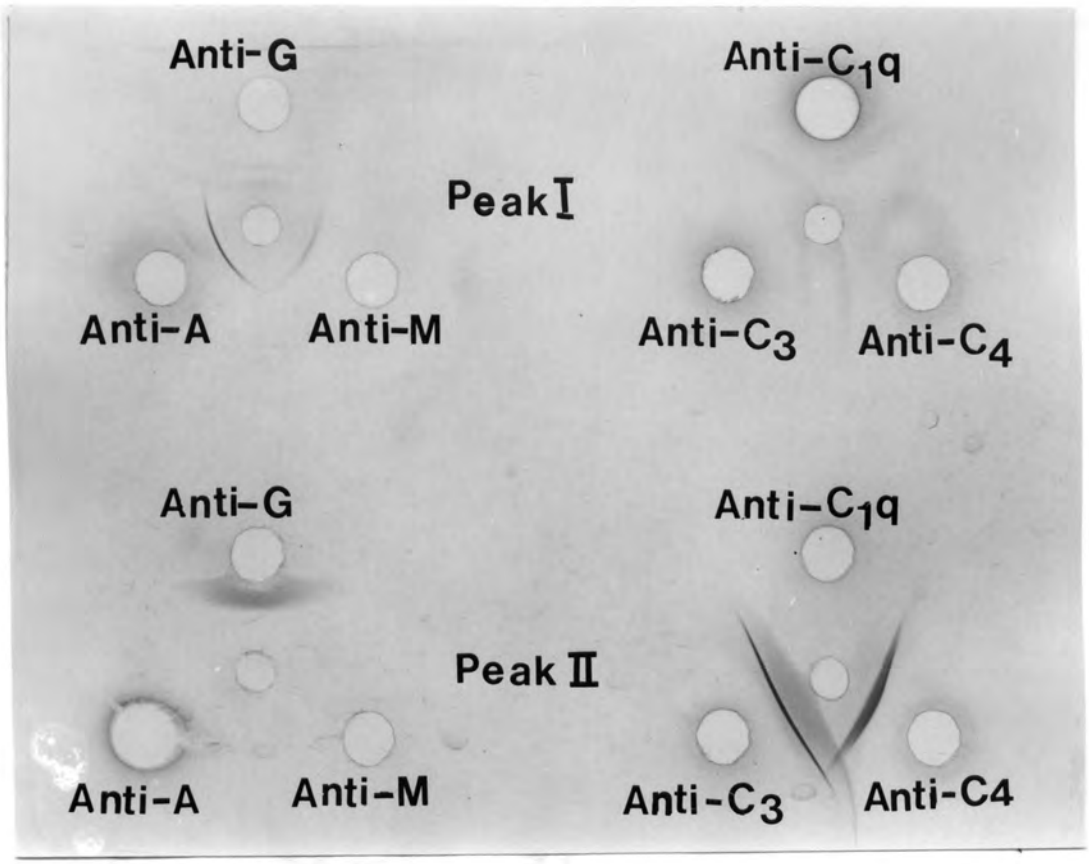


Figure 13

Elution profile of the separation on an acid ACA-34 column of the Protein A bound fraction from the high molecular weight peak of a sera from a patient with severe and chronic mucocutaneous candidiasis.

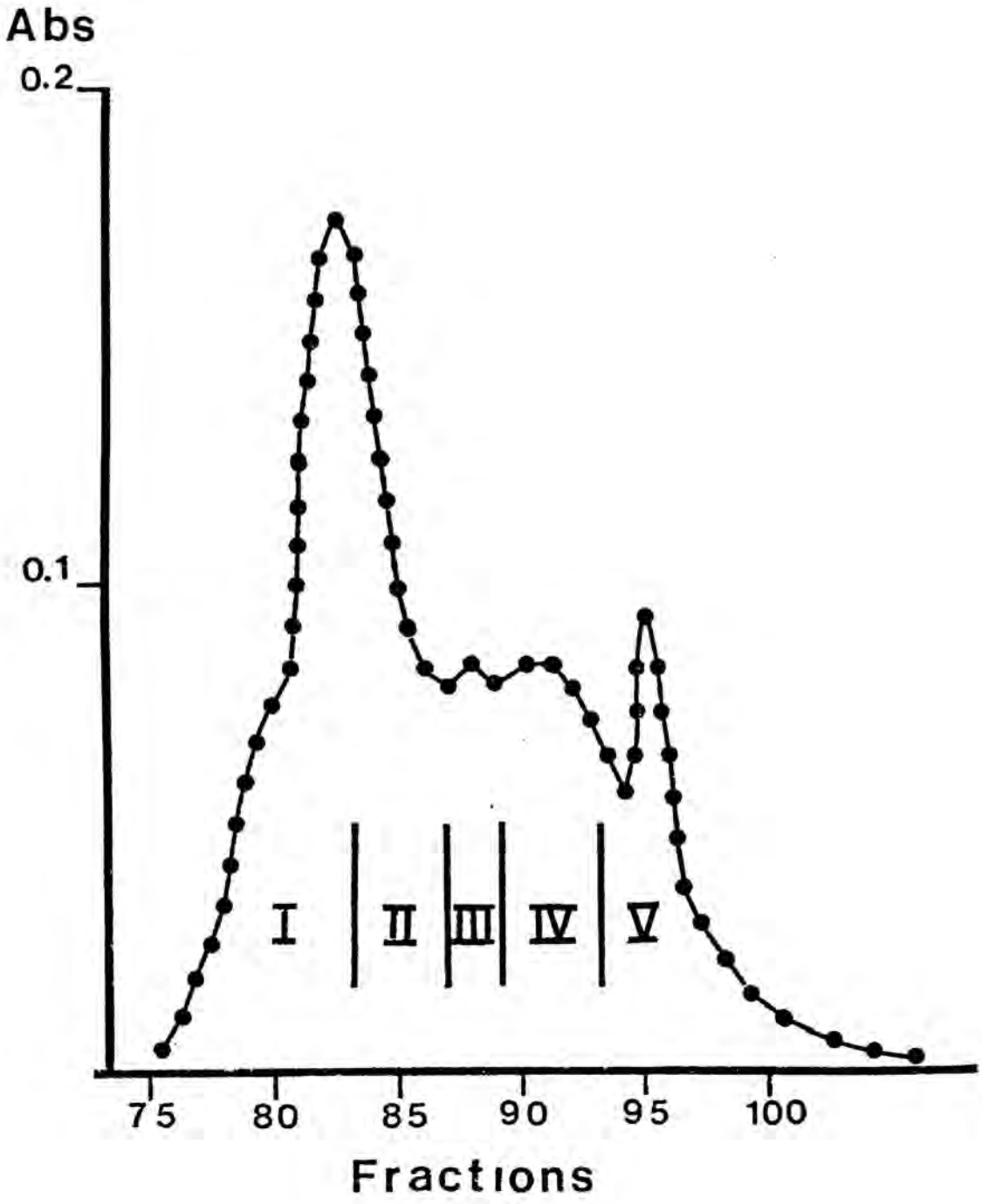


Figure 14

Counterimmunoelectrophoresis plate showing the detection of antigen in the eluted fractions from the acid-ACA-34 separation of the patient with severe and chronic mucocutaneous candidiasis.

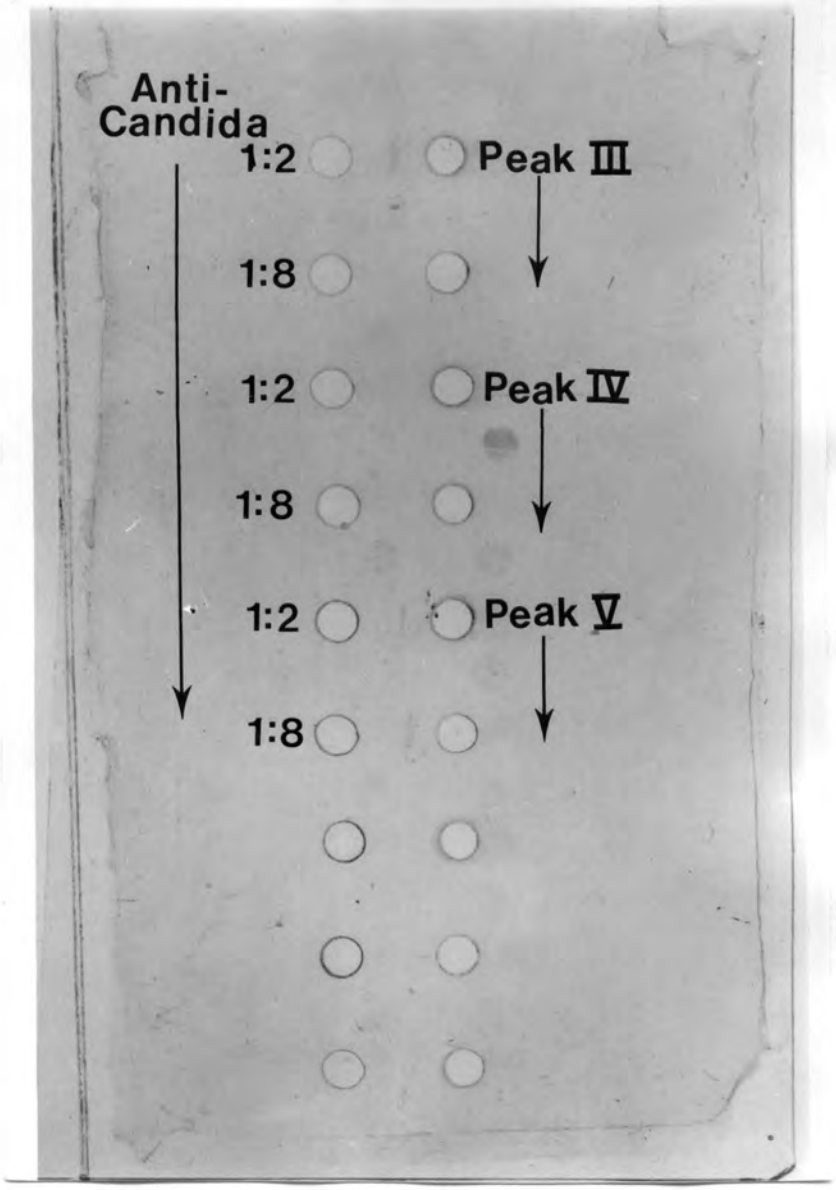


plate can be seen in Figure 14.

The results of the data on separation and characterization of immune complexes, though not totally consistent with respect to the finding of antigen and antibody in high molecular weight fractions of all sera, certainly suggests that complexes consisting of Candida antigen and antibody do exist in the sera of some patients.

Discussion

The value of the serological methods of diagnosing disseminated candidiasis has been a point of controversy since the first reports of detection of anti-*Candida* antibodies in patients with systemic disease prompted hopes that the presence of these antibodies could lead to a definitive diagnosis. Initially, the most commonly used tests were for agglutinating antibodies. Agglutinins are found in a high percentage of individuals with systemic candidiasis but they also occur in a large percentage of individuals without evidence of *Candida* infection. Winner, in 1955, (91) found agglutinins in 31.6% of 2,017 hospital patients and Scuro and Perrone in 1959, (92) found an incidence of 42.1% in 301 subjects without evidence of candidiasis. Agglutinating antibodies may be found in even higher percentages of patients with superficial forms of *Candida* infection. 80% of patients with chronic mucocutaneous candidiasis were found by Lehner et al. (42) to be positive when tested for *Candida* agglutinins. Complement-fixing antibodies have likewise been found in high percentages of normals and superficially infected patients. In the group of 301 normals tested by Scuro and Perrone (92), 30.6% were positive by complement fixation and in a group

of 48 patients with Candida skin infections tested by Peck et al. (33), 75% were found to be positive.

Indirect fluorescent antibody to Candida has been found less frequently in normal individuals. In six series of healthy donors (39, 93, 94, 95, 96, 97), only two (39, 97) reported any incidence of positive reaction and those were 18.8% and 16.0% respectively. Among patients with superficial forms of candidiasis, however, percentages were much higher - 64% in a series of 75 patients with oral candidiasis (93) and 51.4% in a series of 37 with oral infections (95).

Precipitating antibodies have generally been found to be more specific for systemic candidiasis. A number of authors (39, 94, 97, 98) claim to have found no positive reactors in normal individuals. Others, however, have found a low incidence - 10.4% of 604 individuals in a study by Douchet and Muller (99) and 3.7% of 191 individuals in a study by Everall et al. (100). In patients with superficial forms of candidiasis, again, the incidence of positive reactors is high in some studies. Stanley and Hurley (101) found 34.4% of 125 pregnant women with vaginal candidiasis to have Candida precipitins in their sera. Taschdjian et al. (102) found an incidence of 25.5% positives in 51 subjects with superficial candidiasis.

Questioning the value of these antibody detection tests, therefore, appears to be justified in light of their lack of specificity for systemic candidiasis. A

better means of identifying the individuals with deep-seated infection, who must be treated aggressively, is obviously needed.

Several authors have noted that quantitation of antibody concentration may be a means of achieving greater differentiation between groups with and without systemic candidiasis. In agglutination, complement fixation and fluorescent antibody assays, results are usually reported in terms of titers. Seeliger (103) noted that agglutinins found in healthy individuals rarely exceed a titer of 1:160. Coudert et al. (94) failed to demonstrate titers greater than 1:40 in 217 individuals including 17 with superficial candidiasis and 62 with noncandidal infections. Taschdjian et al. (3) have summarized the results of five studies (39, 42, 93, 94, 104) with indirect fluorescent antibody and found that titers greater than 1:160 are rarely encountered in healthy individuals.

The development of the quantitative immunofluorescence assay provided a means of objective measurement of anti-Candida antibodies whereby the hypothesis could be tested that higher titers of antibody are associated with systemic disease, whereas lower titers are found associated with superficially infected patients and normal individuals (45). Results in two series of patients included in these studies suggest that this hypothesis is valid. In the series of patients tested in the initial studies where a commercial yeast extract was used as antigen, a difference

between mean antibody levels in the group of systemic patients and the group of normal controls was found to be significant at a level of $p < 0.01$. In the series of patients tested against purified cell wall polysaccharide antigens, the QIF assay was able to differentiate between systemic patients and normals and, more importantly, between systemic patients and those with a superficial infection at a level of significance of $p < 0.0005$. If a value greater than two standard deviations above the normal mean is considered as a positive test, none of the normals is positive when tested against either polysaccharide antigen while only one of the superficially infected patients is positive with yeast polysaccharide antigen and three are positive with mycelial polysaccharide.

The QIF assay could, therefore, offer a significant improvement in specificity in the diagnosis of systemic candidiasis through quantitation of antibody.

Another value of the QIF assay lies in its easy adaptation for the detection of class of antibody. We were able to use the assay to test the hypothesis that superficial forms of Candida infection involve the predominant production of IgA antibodies rather than IgG. Earlier studies by Mathur et al. (32) using indirect immunofluorescence on smears of Candida cells to test the sera of vaginal candidiasis patients had suggested that this was the case. Warnock et al. (90), however, using the same technique, disputed these findings and showed that the

distribution of antibody in vaginal candidiasis patients' sera was similar to that in systemic patients - that is, predominantly IgG. Our studies using QIF and indirect immunofluorescence have confirmed the latter findings. The differences in these studies may, possibly, be due to differences in patient selection or to differences in the specificity of the antisera. In any event, it seems evident that the initial impression that the assay of IgA anti-Candida antibodies could be of value in the diagnosis of vaginal candidiasis cannot be sustained in light of the present data.

Another suggested means of differentiating between systemic candidiasis patients and other groups has been the detection of antibodies to purified components of Candida organisms. Observations on the rapid phagocytosis and breakdown of Candida organisms injected intravenously (35, 105), assumedly resulting in the release of Candida cell contents, have prompted the idea that antibodies to cytoplasmic proteins might be more specific for disseminated disease than antibodies to cell wall polysaccharide.

Prior to our efforts, studies using defined antigens have been done using the precipitin reaction, as most agglutination and classical indirect immunofluorescence tests use whole Candida cells as substrate and, thus, detect anti-cell wall mannan antibodies. Taschdjian et al. (3) prepared a Candida extract, called the S antigen, and used it in immunodiffusion. Though the authors have

called it a "cytoplasmic extract", they describe two precipitin lines when the antigen is tested against rabbit sera, one of which could be removed by absorption of the antiserum with Candida spores. This indicates that the extract contains a cell wall component. Precipitins against both antigens were found in 83% of patients diagnosed as having systemic candidiasis but the same reaction was found in patients with chronic cutaneous or mucocutaneous candidiasis associated with autoimmune endocrinopathy. The authors claim that no reaction at all was demonstrated in normals or patients with banal superficial candidiasis. Hellwege et al. (106), using a similar antigen, however, found false positive precipitin reactions in three of 93 healthy individuals and eight of ten patients with bronchial asthma.

Buckley, Lapa, and Hipp (107) removed the polysaccharide from their extract by precipitation with concanavallin A and eliminated the positive precipitin reactions of eight patients with banal superficial candidiasis and three with allergic conditions, while five patients with systemic candidiasis and two with chronic superficial candidiasis reacted with the protein fraction. Syverson et al. (75) found 33 of 271 hospitalized patients without evidence of deep candidal infection, six of 22 vaginal candidiasis patients, and ten of 41 patients with other fungal infections to have positive precipitin reactions with an extract containing polysaccharide. After purifica-

tion of the antigen on Con A, only six of the hospitalized patients, none of the vaginal candidiasis patients, and two of the patients with other fungi reacted.

In our studies we have also shown that precipitin reactions in normals and in patients with cross-reacting fungi are indeed due to the reaction with the polysaccharide component. In contrast, precipitating antibodies to purified cytoplasmic antigen were found only in patients with systemic disease and in one patient with a long-standing chronic mucocutaneous candidiasis. These results confirm that when a qualitative test for precipitating antibodies is used, the use of purified cytoplasmic antigen rather than a crude extract containing cell wall polysaccharide, provides a much more specific test for systemic disease. There is, however, some lack of sensitivity since three of a total of 12 (Series I and II) patients with systemic disease did not have anti-yeast cytoplasmic antibodies and eight of 21 patients did not have anti-mycelial cytoplasmic antibodies. This may be partly due to these patients having been infected with strains of Candida other than albicans as was true with five of our negative patients who had been infected with C. tropicalis. But, since three others were also negative, the difference in strains does not entirely account for the lack of sensitivity. Testing against cytoplasmic extracts from various strains, however, might lead to some improvement.

Our QIF assay has allowed us to combine a quantitative

assay with the use of purified antigens and has resulted in our finding that antigens containing cell wall polysaccharide can be much more valuable in a quantitative than a qualitative assay. Statistically, excellent differentiation between systemic patients and normal or superficially infected patients is obtained with yeast ($p < 0.004$) and mycelial ($p < 0.001$) polysaccharide antigens. The specificity of the test appears better with yeast polysaccharide antigen since only one out of 15 superficially infected patients and no normal has a value greater than two standard deviations above the normal mean. With mycelial polysaccharide, three superficially infected patients have significant levels of antibody. With regard to sensitivity, however, mycelial polysaccharide appears better since 13 of 14 systemic patients have significantly elevated antibody values while nine of 14 are positive with yeast polysaccharide.

When QIF is done using cytoplasmic antigens, the specificity is not as great as it appeared with CIE, possibly because this technique is more sensitive and so detects lower levels of anti-cytoplasmic antibody which may be present in less severely infected individuals. However, all the patients with systemic candidiasis still do not have evidence of anti-cytoplasmic antibodies even with this technique. A compromised level of immunocompetence in these patients could possibly account for this.

In summary, our data on the detection of antibodies

to Candida suggests that both approaches, quantitation of antibody and purification of antigen, can result in improvements in the value of these tests in the diagnosis of systemic candidiasis. If a qualitative precipitin technique is used, the use of cytoplasmic antigens provides a test more specific for systemic disease. If a quantitative test is used, however, the purification of the antigens is less essential as adequate differentiation between deeply infected patients and others can be attained by antigens containing cell wall polysaccharide. In fact, there seems to be more overlap between groups when the cytoplasmic antigens are used in the quantitative technique.

It has also been suggested (45) that, since the mycelial form of Candida albicans is thought to be associated with severe infections as opposed to saprophytic colonization, mycelium specific antigens might also be more specific for disseminated disease. However, our study has not been able to clearly define the value of the mycelial growth phase antigens as opposed to yeast phase antigens. Although more of the systemic patients were found to be positive with mycelial antigens than with yeast antigens, there also were more positive results with these antigens and superficially infected patients. This question remains open for discussion.

It seems apparent that an absolute diagnosis of systemic candidiasis cannot be accomplished through antibody detection techniques even though their value is improved

by quantitation and antigen purification.

Many investigators (51, 52, 53, 54) have, therefore, turned to detection of circulating antigen as a possible means to achieve a more absolute diagnosis. Although some have claimed a degree of success, others (55, 56) have questioned the helpfulness of these assays. Our difficulties in attempting antigen detection lead us to support the latter group.

Several ideas have been offered to explain the difficulties with antigen detection. Lehman and Reiss (55) have suggested that one problem may be the existence of major differences in the sensitivity of anti-mannan sera to the two serotypes of C. albicans mannan. They also have observed temperature sensitivity which may affect the success of the tests, especially when ELISA is used. Studies by Warren et al. (108) showed that antigen was not detectable when the antibody used was anti-mannan IgG from immunized rabbits but that IgG from rabbits convalescing from disseminated candidiasis was capable of detecting antigen in the sera of lethally infected rabbits and mice. This suggests that some antigen other than mannan may be what is detected. Jones (56) suggests that the limits of detection of the available assays may not be adequate to detect extremely small levels of antigen present in the sera of infected individuals since declaring a serum positive becomes extremely difficult when detection of a concentration < 100 ng/ml is necessary. Jones found that

cell wall mannan is cleared very rapidly from the blood and that a disseminated infection with a very large number of organisms ($\geq 10^9$) would be necessary to reliably produce a mannan concentration > 100 ng/ml in the sera of rabbits. When such a large number of organisms is used to induce infection, the inoculum is uniformly lethal in less than 24 hours so that meaningful studies cannot be done. Our work agrees with this since three rabbits injected with 10^9 organisms and one of two infected with 10^8 died within 24 hours. Jones further suggests that $\geq 10^{10}$ Candida would be required to produce > 100 ng/ml of mannan in human serum.

Finally, those authors (53, 57) who have been successful in detecting circulating antigen have often found it necessary to pretreat the sera in such a way that antigen would be released from antibody. Weiner and Coats-Stephen (53) noted that none of 17 rabbit sera not subjected to dissociating conditions had detectable antigen but all had detectable antigen after heat extraction of serum bound antigen. However, our experiments with a sodium hydroxide treatment of mixtures of normal serum and purified antigen indicate that subjection of serum to dissociating conditions can result in denaturation of antigen and interference with detection assays. It seems logical to conclude that milder and safer techniques to dissociate antigen-antibody complexes may need to be developed for antigen detection to become a routine assay.

On the other hand, though much has been assumed about the presence of immune complexes in candidiasis patients' sera, no hard evidence of their existence has been presented until this time. Results of the screening tests which were performed in our group of disseminated candidiasis patients indicated a statistically significant difference did exist in the levels of complexes in this group and a group of normals. Isolation and characterization of the complexes from five patients substantiated the presence of anti-Candida antibody and/or Candida antigen in complexes of at least three of them. In the other two, it was less certain as the antibody or antigen was found in the fraction which did not bind to Protein A rather than in the IgG containing fraction that did bind. However, Candida antigen or antibody was found in the high molecular weight fraction of these sera and antibody might have been of a class other than IgG or of subclass IgG_3 which would not have bound to Protein A. The presence of complement components in the high molecular weight fractions and the Protein A bound fractions of these sera further substantiated the existence of immune complexes.

These results provide support for the view that circulating immune complexes containing microbial antigen do exist in systemic candidiasis and, therefore, may indeed be one reason for difficulties in detecting Candida antigen since a variable fraction of the antigenic determinants of circulating antigen will be "hidden" by binding

to antibody molecules. In addition to their significance with respect to antigen detection, any possible role of immune complexes in the pathogenesis of the disease is unknown. There is no evidence for any immune-complex-mediated vasculitis or glomerulonephritis as is seen in the renal lesions of malaria (109) and in post-streptococcal glomerulonephritis (110). Another possibility, which has been suggested in coccidioidomycosis (10), is that the role of immune complexes might be related to their potential immunosuppressive effects. Immune complexes have been shown to depress T cell-mediated delayed type hypersensitivity (11) and cell-mediated cytotoxicity (112), and to suppress the chemotactic response of polymorphonuclear neutrophils (113). Considering the importance of phagocytic cells of the body in ingesting Candida and of the cellular immune response in defense against fungal infections, it seems possible that immune complex-mediated suppression of the immune response might have an adverse effect on the course of the disease. This is an interesting hypothesis that may be pursued now that the presence of the immune complexes has been established.

In summary, our studies on the immunological approach to the diagnosis of systemic candidiasis have resulted in several findings. The development of a quantitative immunofluorescence method of antibody detection provides an improved diagnostic test for systemic candidiasis. Our studies on the relative merits of cytoplasmic and polysaccha-

ride antigens of the yeast and mycelial phases of Candida growth have supported the idea that cytoplasmic antigens are more valuable in qualitative tests while polysaccharide antigens may be adequate in quantitative tests. Finally, our substantiation of the existence of circulating Candida-anti-Candida immune complexes in the sera of patients with severe candidiasis supports the theory that these immune complexes may be part of the problem with developing a reliable antigen-detection assay and provides a hypothetical mechanism for the suppression of the cellular immune response which may be involved with the progression of the disease.

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